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(54) Title: <b>BONE MORPHOGENIC PROTEIN</b>			
(57) Abstract <p>The present invention relates to novel human BMP polypeptides and isolated nucleic acids containing the coding regions of the genes encoding such polypeptides. Also provided are vectors, host cells, antibodies, and recombinant methods for producing human BMP polypeptides. The invention further relates to diagnostic and therapeutic methods useful for diagnosing and treating disorders related to these novel human BMP polypeptides.</p>			

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## Bone Morphogenic Protein

### *Field of the Invention*

The present invention relates to splice variants of a novel Bone Morphogenic Protein (BMP) gene. More specifically, isolated nucleic acid molecules are provided encoding BMP polypeptides. Amino acid sequences comprising BMP polypeptides are also provided. The present invention further relates to methods and compositions for repairing, reducing or preventing damage to bone, cartilage, and cartilaginous tissues, and for stimulating angiogenesis. The methods and compositions may further be useful for the induction and maintenance of bone and cartilaginous tissue formation, wound healing, and the stimulation and growth of endothelial cells, especially vascular endothelial cells. These methods and compositions may also be useful for augmenting the activity of other compositions useful for the same.

### *Background of the Invention*

The present invention relates to a Bone Morphogenic Protein (BMP) which is responsible for the formation and repair of bone, cartilage, tendon and other tissues present in bone. Members of the bone morphogenetic protein family are useful for induction of cartilage and bone formation. For example, BMP-2 is able to induce the formation of new cartilage and/or bone tissue in vivo in a rat ectopic implant model (See, e.g., U.S. Pat. No: 5,013,649); in mandibular defects in dogs (See, e.g., Toriumi et al., Arch. Otolaryngol Head Neck Surg., 117:1101-1112 (1991)); in femoral segmental defects in sheep (see Gerhart et al., Trans Orthop Res Soc, 16:172 (1991)). Other members of the BMP family also have osteogenic activity, including BMP-4, -6 and -7 (See, e.g., Wozney, Bone Morphogenetic Proteins and Their Gene Expression, in Cellular and Molecular Biology of Bone, pp. 131-167 (Academic Press, Inc. 1993)). BMP proteins further demonstrate inductive and/or differentiation potentiating activity on a variety of other tissues, including cartilage, tendon, ligament, neural tissue.

BMPs form part of the large superfamily of TGF- $\beta$ . (Transforming Growth Factor - $\beta$ ), a family that includes embryonic morphogens, endocrine function regulators, wide-range regulators, and regulators that are specific for cell proliferation and differentiation. TGF- $\beta$  is a prototype of this family. It is a dimer of two identical chains of 112 amino acids held together by disulfide bridges. Each chain is synthesized starting from a longer precursor of about 390 amino acids which has the characteristics of a secretory polypeptide, presenting a hydrophobic sequence in the N-terminal region which should function as a secretory peptide for the secretion of the molecule. The precursor is then processed to its mature form by cleavage by a specific peptidase, which cleaves four basic amino acids immediately prior to the biologically active domain. The precursor region plays an essential role in the correct folding of the mature portion in vivo, to the extent that to date, no mature, biologically active peptides are known to have been produced in *Escherichia coli* by recombinant DNA techniques.

BMPs are known in various animal species from *Drosophila* to humans, their sequences having been maintained to a great extent throughout evolution. The sequence homology among the various polypeptides is usually high, especially in the C-terminal region. The degree of identity of sequence varies between 25 and 90% among the various family members. In the region of homology, between 7 and 9 cysteines are usually conserved among the members. These are involved in the formation of disulfide bridges between the amino-acid chains. BMPs induce chemotactic, proliferative and differential responses, which culminate in the transient formation of cartilage, followed by the accumulation of bone with hematopoietic marrow.

The activity of BMPs is linked with the demineralized bone matrix, and is extractable with denaturing agents. BMPs have been extracted from various species including humans, monkeys, cattle, rats and mice (Sampath, T. K., Reddi, A. H. 1983, PNAS 80,6591-6595; Urist, M. D. et al. 1979, PNAS 76, 1828-1832). Most studies were carried out on BMPs derived from bovine bone, an abundant and easily obtainable source. In 1988 Wozney et al. (Wozney, J. M. et al., 1988, Science 242, 1528-1534) recovered a biologically active protein fraction of about 30 kD from



bovine bone that could be detected by polyacrylamide gel electrophoresis under nonreducing conditions. Following reduction of the disulfide bridges by chemical methods, polypeptides of 30, 18 and 16 kD were obtained (Wang, E. A. et al., 1988, PNAS 85, 9484-9488). This protein fraction was digested with trypsin, and the peptides obtained were separated by HPLC and sequenced. This information was used in the synthesis of DNA probes which were used to identify the bovine genome sequences encoding the various factors. Using portions of these sequences as probes, the human sequences coding for the homologous factors were obtained. Much is now known about these factors (Wozney, J. M. et al., 1990, J. Cell. Sci. Suppl. 13, 149-156; Wozney, J. M., 1989, Progress in Growth Factor Research, 1, 267-280). Some were obtained via recombinant DNA techniques. Some examples of references on growth factors belonging to the above said classes, obtained by recombinant DNA techniques, include EP 409472, WO 9011366, WO 8800205, EP 212474, WO 9105863, and U.S. Pat. NO: 4,743,679.

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### *Summary of the Invention*

The present invention includes isolated nucleic acid molecules comprising a polynucleotide encoding BMP polypeptides. The present invention further includes BMP polypeptides encoded by said polynucleotides. The present invention provides for isolated nucleic acid molecules encoding BMP polypeptides. Further provided for are amino acid sequences comprising BMP polypeptides as disclosed in the sequence listing and encoded by the human cDNA clones described in Table 1 and deposited with the American Type Culture Collection (ATCC) on 22 May, 1998, and given Accession No.: 209889 (HSYAE36) and on 13 July, 1999, and given Accession No.: \_\_\_\_\_ (HETAB62). The ATCC is located at 10801 University Boulevard. Manassas, Virginia 20110-2209.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from the group consisting of : (a) a nucleotide sequence encoding a BMP polypeptide having an amino acid sequence as shown in the sequence listing and described in Table 1; (b) a nucleotide sequence encoding a mature BMP polypeptide having the amino acid

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sequence as shown in the sequence listing and described in Table 1; (c) a nucleotide sequence encoding a biologically active fragment of a BMP polypeptide having an amino acid sequence shown in the sequence listing and described in Table 1; (d) a nucleotide sequence encoding an antigenic fragment of a BMP polypeptide having an amino acid sequence shown in the sequence listing and described in Table 1; (e) a nucleotide sequence encoding a BMP polypeptide comprising the complete amino acid sequence encoded by a human cDNA clone contained in the ATCC Deposit and described in Table 1; (f) a nucleotide sequence encoding a mature BMP polypeptide having an amino acid sequence encoded by a human cDNA clone contained in the ATCC Deposit and described in Table 1; (g) a nucleotide sequence encoding a biologically active fragment of a BMP polypeptide having an amino acid sequence encoded by a human cDNA clone contained in the ATCC Deposit and described in Table 1; (h) a nucleotide sequence encoding an antigenic fragment of a BMP polypeptide having an amino acid sequence encoded by a human cDNA clone contained in the ATCC Deposit and described in Table 1; and (i) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), or (h), above.

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 97%, 98% or 99% identical, to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), or (i) above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide in (a), (b), (c), (d), (e), (f), (g), (h), or (i), above. This polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues. An additional nucleic acid embodiment of the invention relates to an isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a BMP polypeptide having an amino acid sequence in (a), (b), (c), (d), (e), (f), (g), or (h), above.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing

the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of BMP polypeptides or peptides by recombinant techniques. Polypeptides produced by such methods are also provided.

In another aspect, the invention provides isolated polypeptides comprising a  
5 polypeptide having an amino acid sequence described in (a), (b), (c), (d), (e), (f), (g), or (h), above. Polypeptide variants of such BMP polypeptides are also provided.

An additional embodiment of this aspect of the invention relates to a peptide or polypeptide which has the amino acid sequence of an epitope-bearing portion of a BMP polypeptide having an amino acid sequence described herein. Peptides or  
10 polypeptides having the amino acid sequence of an epitope-bearing portion of a BMP polypeptide of the invention include portions of such polypeptides. In another embodiment, the invention provides an isolated antibody that specifically binds a BMP polypeptide having an amino acid sequence described above.

For a number of applications the level of BMP expression can be detected in a  
15 sample of tissue or bodily fluid. The presence of BMP expression or an increased or decreased level of BMP expression can be measured. Thus, the present invention provides for methods useful for detection of BMPs and for the diagnosis of applicable disorders. The diagnosis of disorders involves assaying the expression level of the gene encoding the BMP protein in tissue or bodily fluid from an individual and  
20 comparing the gene expression level with a standard BMP expression level, whereby an increase or decrease in the gene expression level over the standard is indicative of a pathologic disorder, such as arthritis.

The present invention further relates to compositions useful for inducing bone and cartilaginous tissue formation in a patient in need of the same, said compositions  
25 comprising one or more protein members of the present invention.

In other embodiments, the present invention relates to methods for inducing the formation and maintenance of bone and cartilage in a patient, for example a patient suffering from arthritis, particularly osteoarthritis, or a patient with an articular bone or cartilage defect or other bone or cartilaginous tissue defect, said method  
30 comprising administering to said patient an effective amount of a BMP comprising composition. In a particular embodiment, the methods of the present invention relates

to a method for treating articular cartilage defects or damage in a patient in need of the same, said method comprising administering to said patient an effective amount of a BMP comprising composition. The invention further relates to methods for inducing the formation of bone, cartilage and bone and cartilaginous tissue comprising  
5 administering to a patient a BMP comprising composition.

In a further embodiment, the present invention relates to methods for promoting the growth of endothelial cells, and more particularly vascular endothelial cells, and still more particularly for the stimulation of angiogenesis, said method comprising administering to said patient an effective amount of a BMP comprising  
10 composition. In a particular embodiment, the methods of the present invention relates to a method for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions, as well as to stimulate angiogenesis and limb regeneration, said method comprising administering to said patient an effective amount of a BMP  
15 comprising composition..

The methods and compositions of the present invention are thus useful for repairing, reducing, or preventing damage to bone, cartilage, bone and cartilaginous tissue and endothelial tissue. The methods and compositions may further be useful for the induction and maintenance of bone, cartilaginous, and endothelial tissues, wound  
20 healing and other tissue repair, for the induction of bone, cartilaginous, and endothelial tissues (such as articular cartilage, meniscus, the articular surfaces of developing bone, and vascular tissues), and for the treatment of diseases or defects of bone, cartilaginous, and endothelial tissues, such as arthritis, particularly osteoarthritis, and diseases of vascular tissues.

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### ***Detailed Description***

#### **Definitions**

The following definitions are provided to facilitate understanding of certain terms used throughout this specification.

30 In the present invention, "isolated" refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is

altered "by the hand of man" from its natural state. For example, an isolated polynucleotide could be part of a vector or a composition of matter, or could be contained within a cell, and still be "isolated" because that vector, composition of matter, or particular cell is not the original environment of the polynucleotide. The term "isolated" does not refer to genomic or cDNA libraries, whole cell total or mRNA preparations, genomic DNA preparations (including those separated by electrophoresis and transferred onto blots), sheared whole cell genomic DNA preparations or other compositions where the art demonstrates no distinguishing features of the polynucleotide/sequences of the present invention.

As used herein, a "polynucleotide" refers to a molecule having a nucleic acid sequence contained in SEQ ID NO:X (where X may be any of the polynucleotide sequences disclosed in the sequence listing) or a human cDNA contained within a clone deposited with the ATCC and/or described in Table 1. For example, the polynucleotide can contain the nucleotide sequence of the full length cDNA sequence, including the 5' and 3' untranslated sequences, the coding region, with or without a natural or artificial signal sequence, the protein coding region, as well as fragments, epitopes, domains, and variants of the nucleic acid sequence. Moreover, as used herein, a "polypeptide" refers to a molecule having the translated amino acid sequence generated from the polynucleotide as broadly defined.

In the present invention, the sequences identified as SEQ ID NO:X were sometimes generated by overlapping sequences contained in multiple clones (contig analysis). A representative clone containing the entire sequence for SEQ ID NO:X was deposited with the American Type Culture Collection ("ATCC") and/or described in Table 1. As shown in Table 1, each clone is identified by a cDNA Clone ID (Identifier) and the ATCC Deposit Number. The ATCC is located at 10801 University Boulevard, Manassas, Virginia 20110-2209, USA. The ATCC deposit was made pursuant to the terms of the Budapest Treaty on the international recognition of the deposit of microorganisms for purposes of patent procedure.

A "polynucleotide" of the present invention also includes those polynucleotides capable of hybridizing, under stringent hybridization conditions, to sequences contained in SEQ ID NO:X, the complement thereof, or the cDNA within

the clone deposited with the ATCC. "Stringent hybridization conditions" refers to an overnight incubation at 42° C in a solution comprising 50% formamide, 5x SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA,  
5 followed by washing the filters in 0.1x SSC at about 65°C.

In specific embodiments, the polynucleotides of the invention are less than 300 kb, 200 kb, 100 kb, 50 kb, 15 kb, 10 kb, 7.5 kb, 5 kb, 2.5 kb, 2.0 kb, and 1 kb, in length. In a further embodiment, polynucleotides of the invention comprise a portion of the coding sequences, as disclosed herein, but do not comprise all or a portion of  
10 any intron. In another embodiment, the polynucleotides comprising coding sequences do not contain coding sequences of a genomic flanking gene (i.e., 5' or 3' to the BMP gene in the genome).

Also contemplated are nucleic acid molecules that hybridize to the polynucleotides of the present invention at lower stringency hybridization conditions.  
15 Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency); salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 0.2M  
20 NaH<sub>2</sub>PO<sub>4</sub>; 0.02M EDTA, pH 7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA; followed by washes at 50°C with 1XSSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC).

Note that variations in the above conditions may be accomplished through the  
25 inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. Typical blocking reagents include Denhardt's reagent, BLOTTO, heparin, denatured salmon sperm DNA, and commercially available proprietary formulations. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above,  
30 due to problems with compatibility.

Of course, a polynucleotide which hybridizes only to polyA+ sequences (such as any 3' terminal polyA+ tract of a cDNA shown in the sequence listing), or to a complementary stretch of T (or U) residues, would not be included in the definition of "polynucleotide," since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

The polynucleotides of the present invention can be composed of any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, the polynucleotide can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. A polynucleotide may also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

The polypeptides of the present invention can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of

modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., *Meth Enzymol*, 182:626-646 (1990); Rattan et al., *Ann NY Acad Sci.*, 663:48-62 (1992)).

"SEQ ID NO:X" refers to a polynucleotide sequence while "SEQ ID NO:Y" refers to a polypeptide sequence (where Y may be any of the polypeptide sequences disclosed in the sequence listing), both sequences identified by an integer specified in Table 1.

"A polypeptide having biological activity" refers to polypeptides exhibiting activity similar, but not necessarily identical to, a BMP polypeptide of the present invention, including mature forms, as measured in a particular biological assay, with or without dose dependency. In the case where dose dependency does exist, it need not be identical to that of the polypeptide, but rather substantially similar to the dose-dependence in a given activity as compared to the polypeptide of the present invention (i.e., the candidate polypeptide will exhibit greater activity or not more than about 25-fold less and, preferably, not more than about tenfold less activity, and most



preferably, not more than about three-fold less activity relative to the polypeptide of the present invention.)

### **Polynucleotides and Polypeptides of the Invention**

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#### **FEATURES OF PROTEIN ENCODED BY GENE NO: 1**

The translation product of this gene shares sequence homology with Bone Morphogenic Protein (BMP) from both chicken (See Genbank Accession No gil2852121) and human (See International Publication No. WO8800205-A), which  
10 are thought to function in bone, cartilage, and connective tissue formation, and in inducing ectopic bone formation and regulating vertebrate matrix deposition. Therefore, it is expected that the translation product of this clone shares some biological functions with the BMP proteins listed above. The cDNA of gene NO:1, contained in clone HETAB62, is a splice variant of the cDNA contained in clone  
15 HSYAE36. The gene encoding the disclosed cDNA is thought to reside on chromosome 4. Accordingly, polynucleotides related to this invention are useful as a marker in linkage analysis for chromosome 4.

This gene is expressed primarily in osteoclastoma, and to a lesser extent in parathyroid tumor tissue.

20 Therefore, polynucleotides and polypeptides of the invention are useful as reagents for differential identification of the tissue(s) or cell type(s) present in a biological sample and for diagnosis of diseases and conditions which include but are not limited to: disorders of the skeletal, vascular and connective tissues, and parathyroid tumors. Similarly, polypeptides and antibodies directed to these  
25 polypeptides are useful in providing immunological probes for differential identification of the tissue(s) or cell type(s). For a number of disorders of the above tissues or cells, particularly of the skeletal and vascular systems, expression of this gene at significantly higher or lower levels may be routinely detected in certain tissues or cell types (e.g., skeletal, vascular, cancerous and wounded tissues) or bodily  
30 fluids (e.g., lymph, serum, plasma, urine, synovial fluid and spinal fluid) or another tissue or sample taken from an individual having such a disorder, relative to the

standard gene expression level, i.e., the expression level in healthy tissue or bodily fluid from an individual not having the disorder.

Preferred polypeptides of the present invention comprise immunogenic epitopes shown in SEQ ID NO: 4 as residues: Gly-15 to Leu-26, Ser-33 to His-46,  
5 Gln-133 to Asn-138, Asp-214 to Trp-220, Ser-249 to Phe-255, Glu-261 to Asp-267. Further preferred polypeptides comprise amino acid residues: Met-1 to Phe-30, Ser-2 to Phe-30, Gly-40 to Glu-261. Polynucleotides encoding said polypeptides are also provided.

The tissue distribution in osteoclastoma, and the homology to Bone  
10 Morphogenic Proteins (BMPs) from both chicken and human, indicates that polynucleotides and polypeptides corresponding to this gene are useful for the diagnosis and/or treatment of bone disorders. Elevated levels of expression of this gene product in osteoclastoma suggests that it may play a role in the survival, proliferation, and/or growth of osteoclasts. Therefore, it may be useful in influencing  
15 bone mass in such conditions as osteoporosis. Based upon the homology to other BMPs, the translation product of this gene is also useful for the detection and/or treatment of disorders relating to proper formation of bone, cartilage, and connective tissue formation, the induction of ectopic bone formation, and the regulation of vertebrate matrix deposition, and plays a vital role in the regulation of endothelial cell  
20 function; secretion; proliferation; or angiogenesis. An especially preferred use of the polypeptides of the present invention is in the stimulation of angiogenesis. In addition, this gene is useful for the detection and/or treatment of disorders and conditions affecting the connective tissues (e.g. arthritis, trauma, tendonitis, chondromalacia and inflammation), such as in the diagnosis and/or treatment of  
25 various autoimmune disorders such as rheumatoid arthritis, lupus, scleroderma, and dermatomyositis as well as dwarfism, spinal deformation, and specific joint abnormalities as well as chondrodysplasias (ie. spondyloepiphyseal dysplasia congenita, familial arthritis, Atelosteogenesis type II, metaphyseal chondrodysplasia type Schmid). Alternatively, the tissue distribution in parathyroid tumor tissue  
30 suggests that the translation product of this gene is useful for the detection, diagnosis, and/or treatment of tumors of the parathyroid, as well as cancers of other tissues

where expression of this gene has been observed. Protein, as well as, antibodies directed against the protein may show utility as a tumor marker and/or immunotherapy targets for the above listed tissues.

Many polynucleotide sequences, such as EST sequences, are publicly  
5 available and accessible through sequence databases. Some of these sequences are related to SEQ ID NO:2 and may have been publicly available prior to conception of the present invention. Preferably, such related polynucleotides are specifically excluded from the scope of the present invention. To list every related sequence would be cumbersome. Accordingly, preferably excluded from the present invention  
10 are one or more polynucleotides comprising a nucleotide sequence described by the general formula of a-b, where a is any integer between 1 to 2844 of SEQ ID NO:2, b is an integer of 15 to 2858, where both a and b correspond to the positions of nucleotide residues shown in SEQ ID NO:2, and where b is greater than or equal to a + 14.

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#### **FEATURES OF PROTEIN ENCODED BY GENE NO: 2**

The translation product of this gene shares sequence homology with Bone Morphogenic Protein (BMP) from both chicken (See Genbank Accession No  
20 gil2852121) and human (See International Publication No. WO8800205-A), which are thought to function in bone, cartilage, and connective tissue formation, and in inducing ectopic bone formation and regulating vertebrate matrix deposition. Therefore, it is expected that the translation product of this clone shares some biological functions with the BMP proteins listed above. The cDNA of gene NO:2, contained in the cDNA clone HSYAE36, is a splice variant of the cDNA contained in  
25 clone HETAB62. The gene encoding the disclosed cDNA is thought to reside on chromosome 4. Accordingly, polynucleotides related to this invention are useful as a marker in linkage analysis for chromosome 4.

This gene is expressed primarily in bone marrow and osteoclastoma, and to a lesser extent in parathyroid tumor tissue, prostate and lung tissues.

30 Therefore, nucleic acids of the invention are useful as reagents for differential identification of the tissue(s) or cell type(s) present in a biological sample and for

diagnosis of the following diseases and conditions: disorders affecting the skeletal, connective tissue, and vascular systems and hematopoiesis, including osteosarcoma. Similarly, polypeptides and antibodies directed to those polypeptides are useful to provide immunological probes for differential identification of the tissue(s) or cell type(s). For a number of disorders of the above tissues or cells, particularly of the skeletal, vascular, immune, and hematopoietic systems, expression of this gene at significantly higher or lower levels may be detected in certain tissues or cell types (e.g., skeletal, immune, vascular, hematopoietic, cancerous and wounded tissues) or bodily fluids (e.g., lymph, serum, plasma, urine, synovial fluid or spinal fluid) taken from an individual having such a disorder, relative to the standard gene expression level, i.e., the expression level in healthy tissue from an individual not having the disorder.

Preferred polypeptides of the present invention comprise immunogenic epitopes shown in SEQ ID NO: 5 as residues: Gly-15 to Glu-23, Ala-34 to Thr-39, Arg-51 to His-57, Gly-60 to His-66, Gln-153 to Asn-158, Asp-234 to Trp-240, Ser-269 to Asn-274, Glu-281 to Phe-290. Further preferred polypeptides comprise amino acid residues: Met-1 to Phe-32, Ser-2 to Phe-32, Gly-41 to Glu-281. Polynucleotides encoding said polypeptides are also provided.

The tissue distribution and homology to BMPs suggests that the protein product of this clone is useful for the diagnosis and/or treatment of disorders affecting the skeletal system and hematopoiesis, including osteosarcomas. Elevated levels of expression of this gene product in osteoclastoma suggests that it may play a role in the survival, proliferation, and/or growth of osteoclasts. Therefore, it may be useful in influencing bone mass in such conditions as osteoporosis. Based upon the homology to other BMPs and other scientific data, the translation product of this gene is also useful for the detection and/or treatment of disorders relating to proper formation of bone, cartilage, and connective tissue formation, the induction of ectopic bone formation, and the regulation of vertebrate matrix deposition, or it may play a vital role in the regulation of endothelial cell function; secretion; proliferation; or angiogenesis. An especially preferred use of the polypeptides of the present invention is in the stimulation of angiogenesis. In addition, this gene is useful for the detection

and/or treatment of disorders and conditions affecting the connective tissues (e.g. arthritis, trauma, tendonitis, chondromalacia and inflammation), such as in the diagnosis and/or treatment of various autoimmune disorders such as rheumatoid arthritis, lupus, scleroderma, and dermatomyositis as well as dwarfism, spinal  
5 deformation, and specific joint abnormalities as well as chondrodysplasias (ie. spondyloepiphyseal dysplasia congenita, familial arthritis, Atelosteogenesis type II, metaphyseal chondrodysplasia type Schmid). Alternatively, the tissue distribution in parathyroid tumor tissue suggests that the translation product of this gene is useful for the detection, diagnosis, and/or treatment of tumors of the parathyroid, as well as  
10 cancers of other tissues where expression of this gene has been observed. Protein, as well as, antibodies directed against the protein may show utility as a tumor marker and/or immunotherapy targets for the above listed tissues.

Many polynucleotide sequences, such as EST sequences, are publicly available and accessible through sequence databases. Some of these sequences are  
15 related to SEQ ID NO:3 and may have been publicly available prior to conception of the present invention. Preferably, such related polynucleotides are specifically excluded from the scope of the present invention. To list every related sequence would be cumbersome. Accordingly, preferably excluded from the present invention are one or more polynucleotides comprising a nucleotide sequence described by the  
20 general formula of a-b, where a is any integer between 1 to 2780 of SEQ ID NO:3, b is an integer of 15 to 2794, where both a and b correspond to the positions of nucleotide residues shown in SEQ ID NO:3, and where b is greater than or equal to a + 14.

**TABLE 1**

Gene No.	cDNA Clone ID	ATCC Deposit Nr and Date	Vector	NT SEQ ID NO: X	Total NT Seq.	5' NT of Clone Seq.	3' NT of Clone Seq.	5' NT of Start Codon	5' NT of First AA of Signal Pep	AA SEQ ID NO: Y	First AA of Sig Pep	Last AA of Sig Pep	First AA of Secreted Portion	Last AA of ORF
1	HETAB62	07/13/99	Uni-ZAP XR	2	2858	1	2858	327	327	4	1	22	23	345
2	HSYAE36	209889 5/22/98	pCMVSPORT 3.0	3	2794	1	2794	203	203	5	1	22	23	297

Table 1 summarizes the information corresponding to each "Gene NO:" described above. The nucleotide sequence identified as "NT SEQ ID NO:X" was assembled from partially homologous ("overlapping") sequences obtained from the "cDNA clone ID" identified in Table 1 and, in some cases, from additional related DNA  
5 clones. The overlapping sequences were assembled into a single contiguous sequence of high redundancy (usually three to five overlapping sequences at each nucleotide position), resulting in a final sequence identified as SEQ ID NO:X.

The cDNA Clone ID was deposited on the date and given the corresponding deposit number listed in "ATCC Deposit No:Z and Date." Some of the deposits  
10 contain multiple different clones corresponding to the same gene. "Vector" refers to the type of vector contained in the cDNA Clone ID.

"Total NT Seq." refers to the total number of nucleotides in the contig identified by "Gene NO:" The deposited clone contains all of these sequences, reflected by the nucleotide position indicated as "5' NT of Clone Seq." and the "3'  
15 NT of Clone Seq." of SEQ ID NO:X. The nucleotide position of SEQ ID NO:X of the putative methionine start codon (if present) is identified as "5' NT of Start Codon." Similarly, the nucleotide position of SEQ ID NO:X of the predicted signal sequence (if present) is identified as "5' NT of First AA of Signal Pep."

The translated amino acid sequence, beginning with the first translated codon  
20 of the polynucleotide sequence, is identified as "AA SEQ ID NO:Y," although other reading frames can also be easily translated using known molecular biology techniques. The polypeptides produced by these alternative open reading frames are specifically contemplated by the present invention.

SEQ ID NO:X (where X may be any of the polynucleotide sequences  
25 disclosed in the sequence listing) and the translated SEQ ID NO:Y (where Y may be any of the polypeptide sequences disclosed in the sequence listing) are sufficiently accurate and otherwise suitable for a variety of uses well known in the art and described further below. For instance, SEQ ID NO:X is useful for designing nucleic acid hybridization probes that will detect nucleic acid sequences contained in SEQ ID  
30 NO:X or the cDNA contained in the deposited clone. These probes will also hybridize to nucleic acid molecules in biological samples, thereby enabling a variety

of forensic and diagnostic methods of the invention. Similarly, polypeptides identified from SEQ ID NO:Y may be used to generate antibodies, which bind specifically to the secreted proteins encoded by the cDNA clones identified in Table 1.

5           Nevertheless, DNA sequences generated by sequencing reactions can contain sequencing errors. The errors exist as misidentified nucleotides, or as insertions or deletions of nucleotides in the generated DNA sequence. The erroneously inserted or deleted nucleotides cause frame shifts in the reading frames of the predicted amino acid sequence. In these cases, the predicted amino acid sequence diverges from the  
10          actual amino acid sequence, even though the generated DNA sequence may be greater than 99.9% identical to the actual DNA sequence (for example, one base insertion or deletion in an open reading frame of over 1000 bases).

            Accordingly, for those applications requiring precision in the nucleotide sequence or the amino acid sequence, the present invention provides not only the  
15          generated nucleotide sequence identified as SEQ ID NO:X and the predicted translated amino acid sequence identified as SEQ ID NO:Y, but also a sample of plasmid DNA containing a human cDNA of the invention deposited with the ATCC, as set forth in Table 1. The nucleotide sequence of each deposited clone can readily be determined by sequencing the deposited clone in accordance with known methods.  
20          The predicted amino acid sequence can then be verified from such deposits. Moreover, the amino acid sequence of the protein encoded by a particular clone can also be directly determined by peptide sequencing or by expressing the protein in a suitable host cell containing the deposited human cDNA, collecting the protein, and determining its sequence.

25           The present invention also relates to the genes corresponding to SEQ ID NO:X, SEQ ID NO:Y, or the deposited clone. The corresponding gene can be isolated in accordance with known methods using the sequence information disclosed herein. Such methods include preparing probes or primers from the disclosed sequence and identifying or amplifying the corresponding gene from appropriate  
30          sources of genomic material.



Also provided in the present invention are alleles species homologs. Alleles and species homologs may be isolated and identified by making suitable probes or primers from the sequences provided herein and screening a suitable nucleic acid source for the desired homologue.

5       The polypeptides of the invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

10       The polypeptides may be in the form of the secreted protein, including the mature form, or may be a part of a larger protein, such as a fusion protein (see below). It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification, such as multiple histidine residues, or an additional sequence for stability during  
15 recombinant production.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of a polypeptide, including the secreted polypeptide, can be substantially purified by the one-step method described in Smith and Johnson, Gene 67:31-40  
20 (1988). Polypeptides of the invention also can be purified from natural or recombinant sources using antibodies of the invention raised against the secreted protein in methods which are well known in the art.

The following procedures can be used to obtain full length genes or full length coding portions of BMPs using the information from the sequences disclosed herein  
25 or the clones deposited with the ATCC.

#### RACE Protocol For Recovery of Full-Length Genes

Partial cDNA clones can be made full-length by utilizing the rapid amplification of cDNA ends (RACE) procedure described in Frohman, M.A., Dush,  
30 M.K. and Martin, G.R. (1988) Proc. Nat'l. Acad. Sci. USA, 85:8998-9002. A cDNA clone missing either the 5' or 3' end can be reconstructed to include the absent base

pairs extending to the translational start or stop codon, respectively. In some cases, cDNAs are missing the start of translation, therefor. The following briefly describes a modification of this original 5' RACE procedure. Poly A+ or total RNA is reverse transcribed with Superscript II (Gibco/BRL) and an antisense or complementary primer specific to the cDNA sequence. The primer is removed from the reaction with a Microcon Concentrator (Amicon). The first-strand cDNA is then tailed with dATP and terminal deoxynucleotide transferase (Gibco/BRL). Thus, an anchor sequence is produced which is needed for PCR amplification. The second strand is synthesized from the dA-tail in PCR buffer, Taq DNA polymerase (Perkin-Elmer Cetus), an oligo-dT primer containing three adjacent restriction sites (XhoI, SalI and ClaI) at the 5' end and a primer containing just these restriction sites. This double-stranded cDNA is PCR amplified for 40 cycles with the same primers as well as a nested cDNA-specific antisense primer. The PCR products are size-separated on an ethidium bromide-agarose gel and the region of gel containing cDNA products the predicted size of missing protein-coding DNA is removed. cDNA is purified from the agarose with the Magic PCR Prep kit (Promega), restriction digested with XhoI or SalI, and ligated to a plasmid such as pBluescript SKII (Stratagene) at XhoI and EcoRV sites. This DNA is transformed into bacteria and the plasmid clones sequenced to identify the correct protein-coding inserts. Correct 5' ends are confirmed by comparing this sequence with the putatively identified homologue and overlap with the partial cDNA clone. Similar methods known in the art and/or commercial kits are used to amplify and recover 3' ends.

Several quality-controlled kits are available for purchase. Similar reagents and methods to those above are supplied in kit form from Gibco/BRL for both 5' and 3' RACE for recovery of full length genes. A second kit is available from Clontech which is a modification of a related technique, SLIC (single-stranded ligation to single-stranded cDNA), developed by Dumas et al. (Dumas, J.B., Edwards, M., Delort, J. and Mallet, J., 1991, Nucleic Acids Res., 19:5227-5232). The major differences in procedure are that the RNA is alkaline hydrolyzed after reverse transcription and RNA ligase is used to join a restriction site-containing anchor

primer to the first-strand cDNA. This obviates the necessity for the dA-tailing reaction which results in a polyT stretch that is difficult to sequence past.

An alternative to generating 5' or 3' cDNA from RNA is to use cDNA library double-stranded DNA. An asymmetric PCR-amplified antisense cDNA strand is  
5 synthesized with an antisense cDNA-specific primer and a plasmid-anchored primer. These primers are removed and a symmetric PCR reaction is performed with a nested cDNA-specific antisense primer and the plasmid-anchored primer.

10 RNA Ligase Protocol For Generating The 5' or 3' End Sequences To Obtain Full Length Genes

Once a gene of interest is identified, several methods are available for the identification of the 5' or 3' portions of the gene which may not be present in the original cDNA clone. These methods include but are not limited to filter probing, clone enrichment using specific probes and protocols similar and identical to 5' and  
15 3'RACE. While the full length gene may be present in the library and can be identified by probing, a useful method for generating the 5' or 3' end is to use the existing sequence information from the original cDNA to generate the missing information. A method similar to 5'RACE is available for generating the missing 5' end of a desired full-length gene. (This method was published by Fromont-Racine et  
20 al., Nucleic Acids Res., 21(7):1683-1684 (1993)). Briefly, a specific RNA oligonucleotide is ligated to the 5' ends of a population of RNA presumably containing full-length gene RNA transcript and a primer set containing a primer specific to the ligated RNA oligonucleotide and a primer specific to a known sequence of the gene of interest, is used to PCR amplify the 5' portion of the desired  
25 full length gene which may then be sequenced and used to generate the full length gene. This method starts with total RNA isolated from the desired source, poly A RNA may be used but is not a prerequisite for this procedure. The RNA preparation may then be treated with phosphatase if necessary to eliminate 5' phosphate groups on degraded or damaged RNA which may interfere with the later RNA ligase step. The  
30 phosphatase if used is then inactivated and the RNA is treated with tobacco acid pyrophosphatase in order to remove the cap structure present at the 5' ends of

messenger RNAs. This reaction leaves a 5' phosphate group at the 5' end of the cap cleaved RNA which can then be ligated to an RNA oligonucleotide using T4 RNA ligase. This modified RNA preparation can then be used as a template for first strand cDNA synthesis using a gene specific oligonucleotide. The first strand synthesis -  
5 reaction can then be used as a template for PCR amplification of the desired 5' end using a primer specific to the ligated RNA oligonucleotide and a primer specific to the known sequence of the BMP of interest. The resultant product is then sequenced and analyzed to confirm that the 5' end sequence belongs to the BMP.

#### 10 **Polynucleotide and Polypeptide Variants**

"Variant" refers to a polynucleotide or polypeptide differing from the polynucleotide or polypeptide of the present invention, but retaining essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the polynucleotide or polypeptide of the present invention.

15 By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the  
20 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. The query sequence may be an entire  
25 sequence shown in Table 1, the ORF (open reading frame), or any fragment specified as described herein.

Other methods for determining and defining, whether any particular nucleic acid molecule or polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of the present invention can be determined conventionally  
30 using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a

subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by  
5 converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject  
10 nucleotide sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject  
15 sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then  
20 subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the  
25 purposes of manually adjusting the percent identity score.

For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a  
matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent  
30 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score

calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject  
5 sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

10 By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other  
15 words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, (indels) or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those  
20 terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

Other methods for determining and defining, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequences shown in the sequence listing or to the amino acid sequence  
25 encoded by deposited DNA clone can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App.  
30 Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said

global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100

residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal  
5 ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to be made for the purposes of the present invention.

The variants may contain alterations in the coding regions, non-coding regions, or both. Especially preferred are polynucleotide variants containing  
10 alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. Nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code are preferred. Moreover, variants in which 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or added in any combination are also preferred. Polynucleotide variants can be  
15 produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*).

Naturally occurring variants are called "allelic variants," and refer to one of several alternate forms of a gene occupying a given locus on a chromosome of an  
20 organism. (Genes II, Lewin, B., ed., John Wiley & Sons, New York (1985).) These allelic variants can vary at either the polynucleotide and/or polypeptide level and are included in the present invention. Alternatively, non-naturally occurring variants may be produced by mutagenesis techniques or by direct synthesis.

Using known methods of protein engineering and recombinant DNA  
25 technology, variants may be generated to improve or alter the characteristics of the polypeptides of the present invention. For instance, one or more amino acids can be deleted from the N-terminus or C-terminus of the secreted protein without substantial loss of biological function. The authors of Ron et al., *J. Biol. Chem.*, 268: 2984-2988 (1993), reported variant KGF proteins having heparin binding activity even after  
30 deleting 3, 8, or 27 amino-terminal amino acid residues. Similarly, Interferon gamma exhibited up to ten times higher activity after deleting 8-10 amino acid residues from



the carboxy terminus of this protein. (Dobeli et al., *J. Biotechnology*, 7:199-216 (1988)).

Moreover, ample evidence demonstrates that variants often retain a biological activity similar to that of the naturally occurring protein. For example, Gayle and  
5 coworkers (*J. Biol. Chem* 268:22105-22111 (1993)) conducted extensive mutational analysis of human cytokine IL-1a. They used random mutagenesis to generate over 3,500 individual IL-1a mutants that averaged 2.5 amino acid changes per variant over the entire length of the molecule. Multiple mutations were examined at every possible amino acid position. The investigators found that "[m]ost of the molecule  
10 could be altered with little effect on either [binding or biological activity]." (See, Abstract.) In fact, only 23 unique amino acid sequences, out of more than 3,500 nucleotide sequences examined, produced a protein that significantly differed in activity from wild-type.

Furthermore, even if deleting one or more amino acids from the N-terminus or  
15 C-terminus of a polypeptide results in modification or loss of one or more biological functions, other biological activities may still be retained. For example, the ability of a deletion variant to induce and/or to bind antibodies which recognize the secreted form will likely be retained when less than the majority of the residues of the secreted form are removed from the N-terminus or C-terminus. Whether a particular  
20 polypeptide lacking N- or C-terminal residues of a protein retains such immunogenic activities can readily be determined by routine methods described herein and otherwise known in the art.

Thus, the invention further includes polypeptide variants which show substantial biological activity. Such variants include deletions, insertions,  
25 inversions, repeats, and substitutions selected according to general rules known in the art so as have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. et al., *Science* 247:1306-1310 (1990), wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid sequence to change.

30 The first strategy exploits the tolerance of amino acid substitutions by natural selection during the process of evolution. By comparing amino acid sequences in

different species, conserved amino acids can be identified. These conserved amino acids are likely important for protein function. In contrast, the amino acid positions where substitutions have been tolerated by natural selection indicates that these positions are not critical for protein function. Thus, positions tolerating amino acid substitution could be modified while still maintaining biological activity of the protein.

The second strategy uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene to identify regions critical for protein function. For example, site directed mutagenesis or alanine-scanning mutagenesis (introduction of single alanine mutations at every residue in the molecule) can be used. (Cunningham and Wells, Science 244:1081-1085 (1989).) The resulting mutant molecules can then be tested for biological activity.

As the authors state, these two strategies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at certain amino acid positions in the protein. For example, most buried (within the tertiary structure of the protein) amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Moreover, tolerated conservative amino acid substitutions involve replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly.

Besides conservative amino acid substitution, variants of the present invention include (i) substitutions with one or more of the non-conserved amino acid residues, where the substituted amino acid residues may or may not be one encoded by the genetic code, or (ii) substitution with one or more of amino acid residues having a substituent group, or (iii) fusion of the mature polypeptide with another compound, such as a compound to increase the stability and/or solubility of the polypeptide (for example, polyethylene glycol), or (iv) fusion of the polypeptide with additional amino

acids, such as an IgG Fc fusion region peptide, or leader or secretory sequence, or a sequence facilitating purification. Such variant polypeptides are deemed to be within the scope of those skilled in the art from the teachings herein.

For example, polypeptide variants containing amino acid substitutions of charged amino acids with other charged or neutral amino acids may produce proteins with improved characteristics, such as less aggregation. Aggregation of pharmaceutical formulations both reduces activity and increases clearance due to the aggregate's immunogenic activity. (Pinckard et al., Clin. Exp. Immunol. 2:331-340 (1967); Robbins et al., Diabetes 36: 838-845 (1987); Cleland et al., Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377 (1993).)

A further embodiment of the invention relates to a polypeptide which comprises the amino acid sequence of a BMP polypeptide having an amino acid sequence which contains at least one amino acid substitution, but not more than 50 amino acid substitutions, even more preferably, not more than 40 amino acid substitutions, still more preferably, not more than 30 amino acid substitutions, and still even more preferably, not more than 20 amino acid substitutions. Of course, in order of ever-increasing preference, it is highly preferable for a peptide or polypeptide to have an amino acid sequence which comprises the amino acid sequence of a BMP polypeptide, which contains at least one, but not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid substitutions. In specific embodiments, the number of additions, substitutions, and/or deletions in the amino acid sequence of Figure 1 or fragments thereof (e.g., the mature form and/or other fragments described herein), is 1-5, 5-10, 5-25, 5-50, 10-50 or 50-150, conservative amino acid substitutions are preferable.

## **Polynucleotide and Polypeptide Fragments**

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the polynucleotide sequences described herein, e.g., shown in the sequence listing or contained in the deposited clones. Uses for the polynucleotide fragments of the present invention include probes, primers, molecular weight, markers, and for expressing the polypeptide fragments of the present invention. Fragments include portions of the polynucleotide sequences, at least 10

contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first, or 5' most, nucleotide for each disclosed polynucleotide sequence is position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotides in length could occupy is included in the invention as an individual specie. "At least" means a fragment may be 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1. Therefore, included in the invention are contiguous fragments specified by any 5' and 3' nucleotide base positions of a polynucleotide sequences wherein the contiguous fragment is any integer between 10 and the length of an entire nucleotide sequence minus 1. The polynucleotide fragments specified by 5' and 3' positions can be immediately envisaged using the clone description and are therefore not individually listed solely for the purpose of not unnecessarily lengthening the specifications. Although it is particularly pointed out that each of the above described species may be included in or excluded from the present invention. The above species of polynucleotides fragments of the present invention may alternatively be described by the formulas "a to b"; where "a" equals the 5' nucleotides position and "b" equals the 3' nucleotides position of the polynucleotide fragment, where "a" equals an integer between 1 and the number of nucleotides of the polynucleotide sequence of the present invention minus 10, where "b" equals an integer between 10 and the number of nucleotides of the polynucleotide sequence of the present invention; and where "a" is an integer smaller than "b" by at least 10.

Again, it is particularly pointed out that each species of the above formula may be specifically included in or excluded from the present invention.

Further, the invention includes polynucleotides comprising sub-genuses of fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from integers between 10 and the length of an entire nucleotide sequence minus 1. Preferred sizes of contiguous nucleotide fragments include 20 nucleotides, 30 nucleotides, 40 nucleotides, 50 nucleotides, 60 nucleotides, 70 nucleotides, 80

nucleotides, 90 nucleotides, 100 nucleotides, 125 nucleotides, 150 nucleotides, 175 nucleotides, 200 nucleotides, 250 nucleotides, 300 nucleotides, 350 nucleotides, 400 nucleotides, 450 nucleotides, 500 nucleotides, 550 nucleotides, 600 nucleotides, 650 nucleotides, 700 nucleotides, 750 nucleotides, 800 nucleotides, 850 nucleotides, 900  
5 nucleotides, 950 nucleotides, 1000 nucleotides. Other preferred sizes of contiguous polynucleotide fragments, which may be useful as diagnostic probes and primers, include fragments 50-300 nucleotides in length which include, as discussed above, fragment sizes representing each integer between 50-300. Larger fragments are also useful according to the present invention corresponding to most, if not all, of the  
10 polynucleotide sequences of the sequence listing or deposited clones. The preferred sizes are, of course, meant to exemplify not limit the present invention as all size fragments, representing any integer between 10 and the length of an entire nucleotide sequence minus 1 of the sequence listing or deposited clones, may be specifically included in or excluded from the invention. Additional preferred nucleic acid  
15 fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of the polypeptides.

The polynucleotide fragments, specified in contiguous nucleotides, can be immediately envisaged using the above description and are therefore not individually listed solely for the purpose of not unnecessarily lengthening the specification.

20 As stated, the present invention also provides for the exclusion of any fragment, specified by 5' and 3' base positions or by size in nucleotide bases as described above for any nucleotide sequence of the sequence listing or deposited clones. Any number of fragments of nucleotide sequences specified by 5' and 3' base positions or by size in nucleotides, as described above, may be specifically excluded  
25 from the present invention.

In the present invention, a "polypeptide fragment" refers to a short amino acid sequence contained in SEQ ID NO:Y or encoded by the cDNA contained in the deposited clone. Protein fragments may be "free-standing," or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a  
30 single continuous region.

Fragments include portions of the amino acid sequences of the sequence

listing and encoded by deposited cDNA clones, at least 7 contiguous amino acid in length, selected from any two integers, one of which representing a N-terminal position and another representing a C-terminal position. The first, or N-terminal most, codon of each polypeptide disclosed herein is position 1. Every combination of  
5 a N-terminal and C-terminal position that a fragment at least 7 contiguous amino acid residues in length could occupy, on any given amino acid sequence is included in the invention as an individual specie. At least means a fragment may be 7 contiguous amino acid residues in length or any integer between 7 and the number of residues in a full length amino acid sequence of the present invention minus 1. Therefore,  
10 included in the invention are species of contiguous fragments specified by any N-terminal and C-terminal positions of amino acid sequence set forth in the sequence listing or encoded by the deposited cDNA clones, wherein the contiguous fragment is any integer between 7 and the number of residues in a full length sequence minus 1. The polypeptide fragments specified by N-terminal and C-terminal positions can be  
15 immediately envisaged using the above description and are therefore not individually listed solely for the purpose of not unnecessarily lengthening the specification. Although it is particularly pointed out that each of the above described species may be specifically included in or excluded from the present invention.

The above species of the polypeptide fragments of the present invention may  
20 alternatively be described by the formula "n to c"; where "n" equals the N-terminal position and "c" sequences the C-terminal position of the polypeptide fragment, where "n" equals an integer between 1 and the number of amino acid residues of the polypeptide sequence of the present invention minus 7, where "c" equals an integer between 7 and the total number of amino acid residues of the polypeptide sequence of  
25 the present invention, and where "n" is an integer smaller than "c" by at least 7.

Again, it is particularly pointed out that each species of the above formula may be specifically included in or excluded from the present invention.

Further, the invention includes polypeptides comprising sub-genuses of fragments specified by size, in amino acid residues, rather than by N-terminal and C-  
30 terminal positions. The invention includes any fragment size, in contiguous amino acid residues, selected from integers between 7 and the number of residues in a full

length sequence minus 1. Preferred sizes of contiguous polypeptide fragments include at least 7 amino acid residues, at least 10 amino acid residues, at least 20 amino acid residues, at least 30 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 75 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, at least 150 amino acid residues, at least 175 amino acid residues, at least 200 amino acid residues, at least 225 amino acid residues, at least 250 amino acid residues, at least 275 amino acid residues, at least 300 amino acid residues, at least 350 amino acid residues, at least 400 amino acid residues, at least 450 amino acid residues, at least 500 amino acid residues, and at least 550 amino acid residues. The preferred sizes are, of course, meant to exemplify, not limit, the present invention as all size fragments representing any integer between 7 and the number of residues in a full length amino acid sequence of the present invention minus 1 are included in the invention.

The contiguous polypeptide fragments specified by size in amino acid residues of the present invention can be immediately envisaged using the above description and are therefore not individually listed solely for the purpose of not unnecessarily lengthening the specification.

The present invention also provides for the exclusion of any fragments specified by N-terminal and C-terminal positions or by size in amino acid residues as described above. Any number of fragments specified by N-terminal and C-terminal positions or by size in amino acid residues as described above.

It is particularly pointed out that the above fragments need not be active since they would be useful, for example, in immunoassays, in epitope mapping, epitope tagging, to generate antibodies to a particular portion of the polypeptide, as vaccines, as molecular weight markers in identifying active biological domains, and in identifying ligand/receptor binding domains.

Also preferred are polypeptide and polynucleotide fragments characterized by structural or functional domains, such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions,

surface-forming regions, substrate binding region, and high antigenic index regions. Polypeptide fragments of SEQ ID NO:Y falling within conserved domains are specifically contemplated by the present invention. Moreover, polynucleotide fragments encoding these domains are also contemplated.

5           Other preferred fragments are biologically active fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of the polypeptide of the present invention. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

10

### **Epitopes & Antibodies**

#### **Epitope-Bearing Portions**

In another aspect, the invention provides peptides and polypeptides  
15 comprising epitope-bearing portions of the polypeptides of the present invention. These epitopes are immunogenic or antigenic epitopes of the polypeptides of the present invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response *in vivo* when the whole polypeptide of the present invention, or fragment thereof, is the immunogen. On the other hand, a region of a  
20 polypeptide to which an antibody can bind is defined as an "antigenic determinant" or "antigenic epitope." The number of *in vivo* immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. *See, e.g.,* Geysen, et al. (1983) Proc. Natl. Acad. Sci. USA 81:3998-4002. However, antibodies can be made to any antigenic epitope, regardless of whether it is an immunogenic epitope, by using  
25 methods such as phage display. *See e.g.,* Petersen G. et al. (1995) Mol. Gen. Genet. 249:425-431. Therefore, included in the present invention are both immunogenic epitopes and antigenic epitopes.

A list of exemplified amino acid sequences comprising immunogenic epitopes of the invention are described herein. It is pointed out that these descriptions only lists  
30 amino acid residues comprising epitopes predicted to have the highest degree of antigenicity using the algorithm of Jameson and Wolf, *Comp. Appl. Biosci.*, 4:181-



186 (1988) (said references incorporated by reference in their entireties). The Jameson-Wolf antigenic analysis was performed using the computer program PROTEAN, using default parameters (Version 3.11 for the Power MacIntosh, DNASTAR, Inc., 1228 South Park Street Madison, WI). Amino acid residues  
5 comprising other immunogenic epitopes may be routinely determined using algorithms similar to the Jameson-Wolf analysis or by *in vivo* testing for an antigenic response using methods known in the art. *See, e.g.,* Geysen et al., *supra*; U.S. Patents 4,708,781; 5, 194,392; 4,433,092; and 5,480,971 (said references incorporated by reference in their entireties).

10 It is particularly pointed out that the described epitopic amino acid sequences comprise immunogenic epitopes. The preferred immunological epitopes of the Features section lists only the critical residues of immunogenic epitopes determined by the Jameson-Wolf analysis. Thus, additional flanking residues on either the N-terminal, C-terminal, or both N- and C-terminal ends may be added to the sequences  
15 to generate an epitope-bearing polypeptide of the present invention. Therefore, the immunogenic epitopes may include additional N-terminal or C-terminal amino acid residues. The additional flanking amino acid residues may be contiguous flanking N-terminal and/or C-terminal sequences from the polypeptides of the present invention, heterologous polypeptide sequences, or may include both contiguous flanking  
20 sequences from the polypeptides of the present invention and heterologous polypeptide sequences.

Polypeptides of the present invention comprising immunogenic or antigenic epitopes are at least 7 amino acids residues in length. "At least" means that a polypeptide of the present invention comprising an immunogenic or antigenic epitope  
25 may be 7 amino acid residues in length or any integer between 7 amino acids and the number of amino acid residues of the full length polypeptides of the invention. Preferred polypeptides comprising immunogenic or antigenic epitopes are at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acid residues in length. However, it is pointed out that each and every integer between 7  
30 and the number of amino acid residues of the full length polypeptide are included in the present invention.

The immunogenic and antigenic epitope-bearing fragments may be specified by either the number of contiguous amino acid residues, or further specified by N-terminal and C-terminal positions of these fragments as described above. Every combination of a N-terminal and C-terminal position that a fragment of at least 7  
5 contiguous amino acid residues in length could occupy on the amino acid sequences of the present invention is included in the invention. Again, "at least 7 contiguous amino acid residues in length" means 7 amino acid residues in length or any integer between 7 amino acids and the number of amino acid residues of the full length polypeptide of the present invention. Specifically, each and every integer between 7  
10 and the number of amino acid residues of the full length polypeptide may be specifically included in or excluded from the present invention.

Immunogenic and antigenic epitope-bearing polypeptides of the invention are useful, for example, to make antibodies which specifically bind the polypeptides of the invention, and in immunoassays to detect the polypeptides of the present  
15 invention. The antibodies are useful, for example, in affinity purification of the polypeptides of the present invention. The antibodies may also routinely be used in a variety of qualitative or quantitative immunoassays, specifically for the polypeptides of the present invention using methods known in the art. *See, e.g.,* Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press; 2nd Ed.  
20 1988).

The epitope-bearing polypeptides of the present invention may be produced by any conventional means for making polypeptides including synthetic and recombinant methods known in the art. For instance, epitope-bearing peptides may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a  
25 simple method for the synthesis of large numbers of peptides, such as 10-20 mgs of 248 individual and distinct 13 residue peptides representing single amino acid variants of a segment of the HA1 polypeptide, all of which were prepared and characterized (by ELISA-type binding studies) in less than four weeks (Houghten, *Proc. Natl. Acad. Sci. USA*, 82:5131-5135 (1985)). This "Simultaneous Multiple  
30 Peptide Synthesis (SMPS)" process is further described in U.S. Patent NO: 4,631,211 to Houghten and coworkers (1986). In this procedure the individual resins for the

solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods. A completely manual procedure allows 500-1000 or more syntheses to be conducted simultaneously (Houghten et al., *Proc. Natl. Acad. Sci.*, 82:5131-5135 at 5134 (1985).

Epitope-bearing polypeptides of the present invention are used to induce antibodies according to methods well known in the art including, but not limited to, *in vivo* immunization, *in vitro* immunization, and phage display methods. See, e.g., Sutcliffe et al., *supra*; Wilson et al., *supra*, and Bittle et al., *J. Gen. Virol.*, 66:2347-2354 (1985). If *in vivo* immunization is used, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine residues may be coupled to a carrier using a linker such as -maleimidobenzoyl- N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carriers using a more general linking agent such as glutaraldehyde. Animals such as rabbits, rats and mice are immunized with either free or carrier-coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 µgs of peptide or carrier protein and Freund's adjuvant. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

As one of skill in the art will appreciate, and discussed above, the polypeptides of the present invention comprising an immunogenic or antigenic epitope can be fused to heterologous polypeptide sequences. For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, any combination thereof including both entire domains and portions thereof) resulting in

chimeric polypeptides. These fusion proteins facilitate purification, and show an increased half-life *in vivo*. This has been shown, *e.g.*, for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. *See*,  
5 *e.g.*, EPA 0,394,827; Traunecker et al., *Nature*, 331:84-86 (1988). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion can also be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone. *See, e.g.*, Fountoulakis et al., *J. Biochem.*, 270:3958-3964 (1988). Nucleic acids encoding the above epitopes can also be recombined with a  
10 gene of interest as an epitope tag to aid in detection and purification of the expressed polypeptide.

### Antibodies

The present invention further relates to antibodies and T-cell antigen receptors  
15 (TCR) which specifically bind the polypeptides of the present invention. The antibodies of the present invention include IgG (including IgG1, IgG2, IgG3, and IgG4), IgA (including IgA1 and IgA2), IgD, IgE, or IgM, and IgY. As used herein, the term "antibody" (Ab) is meant to include whole antibodies, including single-chain whole antibodies, and antigen-binding fragments thereof. Most preferably the  
20 antibodies are human antigen binding antibody fragments of the present invention include, but are not limited to, Fab, Fab' and F(ab')<sub>2</sub>, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), and fragments comprising either a single antigen-binding V<sub>L</sub> or V<sub>H</sub> domain. The antibodies may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine,  
25 rabbit, goat, guinea pig, camel, horse, or chicken.

Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entire or partial of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are any combinations of variable region(s) and hinge region, CH1, CH2,  
30 and CH3 domains. The present invention further includes chimeric, humanized, and human monoclonal and polyclonal antibodies which specifically bind the

polypeptides of the present invention. The present invention further includes antibodies which are anti-idiotypic to the antibodies of the present invention.

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for  
5 different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for heterologous compositions, such as a heterologous polypeptide, small molecule, or solid support material. *See, e.g.*, WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, A. et al., *J. Immunol.*, 147:60-69 (1991); US Patents 5,573,920, 4,474,893, 5,601,819, 4,714,681,  
10 4,925,648; Kostelny et al., *J. Immunol.*, 148:1547-1553 (1992).

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention which are recognized or specifically bound by the antibody. The immunogenic and antigenic epitope(s) or polypeptide portion(s) may be specified as described herein, e.g., by N-  
15 terminal and C-terminal positions, by size in contiguous amino acid residues, or listed in the Tables and Figures. Antibodies which specifically bind any epitope or polypeptide of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind immunogenic and antigenic epitopes of polypeptides of the present invention, and allows for the exclusion of the  
20 same.

Antibodies of the present invention may also be described or specified (i.e., specifically included in or excluded from the present invention) in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homolog of the polypeptides of the present invention may be specifically included in or  
25 excluded from the present invention. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention may also be specifically included in or excluded from the  
30 present invention. Further included in the present invention are antibodies which only bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of

the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified (i.e. specially included in or excluded from the present invention) in terms of their binding affinity. Preferred binding affinities include those with a dissociation constant or  $K_d$  less than  
5  $5 \times 10^{-6}M$ ,  $10^{-6}M$ ,  $5 \times 10^{-7}M$ ,  $10^{-7}M$ ,  $5 \times 10^{-8}M$ ,  $10^{-8}M$ ,  $5 \times 10^{-9}M$ ,  $10^{-9}M$ ,  $5 \times 10^{-10}M$ ,  $10^{-10}M$ ,  $5 \times 10^{-11}M$ ,  $10^{-11}M$ ,  $5 \times 10^{-12}M$ ,  $10^{-12}M$ ,  $5 \times 10^{-13}M$ ,  $10^{-13}M$ ,  $5 \times 10^{-14}M$ ,  $10^{-14}M$ ,  $5 \times 10^{-15}M$ , and  $10^{-15}M$ .

Antibodies of the present invention have uses that include, but are not limited to, methods known in the art to purify, detect, and target the polypeptides of the  
10 present invention including both *in vitro* and *in vivo* diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in biological samples. *See, e.g.,* Harlow et al., *ANTIBODIES: A LABORATORY MANUAL*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) (incorporated by  
15 reference in the entirety).

The antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to polypeptides or other  
20 compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, or toxins. *See, e.g.,* WO 92/08495; WO 91/14438; WO 89/12624; US Patent 5,314,995; and EP 0 396 387.

25 The antibodies of the present invention may be prepared by any suitable method known in the art. For example, a polypeptide of the present invention or an antigenic fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. Monoclonal antibodies can be prepared using a wide of techniques known in the art including the use of hybridoma and recombinant technology. *See, e.g.,* Harlow et al., *ANTIBODIES: A  
30 LABORATORY MANUAL*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988);

Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL  
HYBRIDOMAS 563-681 (Elsevier, N.Y., 1981) (said references incorporated by  
reference in their entireties). Fab and F(ab')<sub>2</sub> fragments may be produced by  
proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or  
5 pepsin (to produce F(ab')<sub>2</sub> fragments).

Alternatively, antibodies of the present invention can be produced through the  
application of recombinant DNA technology or through synthetic chemistry using  
methods known in the art. For example, the antibodies of the present invention can  
be prepared using various phage display methods known in the art. In phage display  
10 methods, functional antibody domains are displayed on the surface of a phage particle  
which carries polynucleotide sequences encoding them. Phage with a desired binding  
property are selected from a repertoire or combinatorial antibody library (e.g. human  
or murine) by selecting directly with antigen, typically antigen bound or captured to a  
solid surface or bead. Phage used in these methods are typically filamentous phage  
15 including fd and M13 with Fab, Fv or disulfide stabilized Fv antibody domains  
recombinantly fused to either the phage gene III or gene VIII protein. Examples of  
phage display methods that can be used to make the antibodies of the present  
invention include those disclosed in Brinkman et al., *J. Immunol. Methods*, 182:41-50  
(1995); Ames et al., *J. Immunol. Methods*, 184:177-186 (1995); Kettleborough et al.,  
20 *Eur. J. Immunol.*, 24:952-958 (1994); Persic et al., *Gene*, 187 9-18 (1997); Burton et  
al., *Advances in Immunology*, 57:191-280 (1994); PCT/GB91/01134; WO 90/02809;  
WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO  
95/20401; and US Patents 5,698,426, 5,223,409, 5,403,484, 5,580,717, 5,427,908,  
5,750,753, 5,821,047, 5,571,698, 5,427,908, 5,516,637, 5,780,225, 5,658,727,  
25 5,733,743 and 5,885,793(said references incorporated by reference in their entireties).

As described in the above references, after phage selection, the antibody  
coding regions from the phage can be isolated and used to generate whole antibodies,  
including human antibodies, or any other desired antigen binding fragment, and  
expressed in any desired host including mammalian cells, insect cells, plant cells,  
30 yeast, and bacteria. For example, techniques to recombinantly produce Fab, Fab' and  
F(ab')<sub>2</sub> fragments can also be employed using methods known in the art such as those

disclosed in WO 92/22324; Mullinax et al., *BioTechniques*, 12(6):864-869 (1994); and Sawai et al., *AJRI*, 34:26-34 (1995); and Better et al., *Science*, 240:1041-1043 (1988) (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and  
5 antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston et al., *Methods in Enzymology*, 203:46-88 (1991); Shu et al., *PNAS*, 90:7995-7999 (1993); and Skerra et al., *Science*, 240:1038-1040 (1988). For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. Methods for producing chimeric  
10 antibodies are known in the art. See e.g., Morrison, *Science*, 229:1202 (1985); Oi et al., *BioTechniques*, 4:214 (1986); Gillies et al., *J. Immunol. Methods*, 125:191-202 (1989); and US Patent 5,807,715. Antibodies can be humanized using a variety of techniques including CDR-grafting (EP 0 239 400; WO 91/09967; US Patent 5,530,101; and 5,585,089), veneering or resurfacing (EP 0 592 106; EP 0 519 596;  
15 Padlan E.A., *Molecular Immunology*, 28(4/5):489-498 (1991); Studnicka et al., *Protein Engineering*, 7(6):805-814 (1994); Roguska et al., *PNAS*, 91:969-973 (1994)), and chain shuffling (US Patent 5,565,332). Human antibodies can be made by a variety of methods known in the art including phage display methods described above. See also, US Patents 4,444,887, 4,716,111, 5,545,806, and 5,814,318; and WO  
20 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741 (said references incorporated by reference in their entireties).

Further included in the present invention are antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations)  
25 to a polypeptide of the present invention. The antibodies may be specific for antigens other than polypeptides of the present invention. For example, antibodies may be used to target the polypeptides of the present invention to particular cell types, either *in vitro* or *in vivo*, by fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or  
30 conjugated to the polypeptides of the present invention may also be used in *in vitro* immunoassays and purification methods using methods known in the art. See e.g.,



Harbor et al. *supra* and WO 93/21232; EP 0 439 095; Naramura et al., *Immunol. Lett.*, 39:91-99 (1994); US Patent 5,474,981; Gillies et al., *PNAS*, 89:1428-1432 (1992); Fell et al., *J. Immunol.*, 146:2446-2452 (1991) (said references incorporated by reference in their entireties).

5           The present invention further includes compositions comprising the polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the hinge  
10 region, CH1 domain, CH2 domain, and CH3 domain or any combination of whole domains or portions thereof. The polypeptides of the present invention may be fused or conjugated to the above antibody portions to increase the *in vivo* half life of the polypeptides or for use in immunoassays using methods known in the art. The polypeptides may also be fused or conjugated to the above antibody portions to form  
15 multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. *See e.g.*, US Patents 5,336,603, 5,622,929,  
20 5,359,046, 5,349,053, 5,447,851, 5,112,946; EP 0 307 434, EP 0 367 166; WO 96/04388, WO 91/06570; Ashkenazi et al., *PNAS*, 88:10535-10539 (1991); Zheng et al., *J. Immunol.*, 154:5590-5600 (1995); and Vil et al., *PNAS*, 89:11337-11341 (1992) (said references incorporated by reference in their entireties).

          The invention further relates to antibodies which act as agonists or antagonists  
25 of the polypeptides of the present invention. For example, the present invention includes antibodies which disrupt the receptor/ligand interactions with the polypeptides of the invention either partially or fully. Included are both receptor-specific antibodies and ligand-specific antibodies. Included are receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation.  
30 Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. Also include are receptor-specific antibodies

which both prevent ligand binding and receptor activation. Likewise, included are neutralizing antibodies which bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included are antibodies which activate the receptor. These antibodies may act as agonists for either all or less than all of the biological activities affected by ligand-mediated receptor activation. The antibodies may be specified as agonists or antagonists for biological activities comprising specific activities disclosed herein. The above antibody agonists can be made using methods known in the art. See *e.g.*, WO 96/40281; US Patent 5,811,097; Deng et al., *Blood*, 92(6):1981-1988 (1998); Chen et al., *Cancer Res.*, 58(16):3668-3678 (1998); Harrop, et al., *J. Immunol.*, 161(4):1786-1794 (1998); Zhu et al., *Cancer Res.*, 58(15):3209-3214 (1998); Yoon et al., *J. Immunol.*, 160(7):3170-3179 (1998); Prat et al., *J. Cell. Sci.*, 111(Pt2):237-247 (1998); Pitard et al., *J. Immunol. Methods*, 205(2):177-190; Liautard et al., *Cytokine*, 9(4):233-241 (1997); Carlson et al., *J. Biol. Chem.*, 272(17):11295-11301 (1997); Taryman et al., *Neuron*, 14(4):755-762 (1995); Muller et al., *Structure*, 6(9):1153-1167 (1998); Bartunek et al., *Cytokine*, 8(1):14-20 (1996) (said references incorporated by reference in their entireties).

## **Fusion Proteins**

Any polypeptide of the present invention can be used to generate fusion proteins. For example, the polypeptide of the present invention, when fused to a second protein, can be used as an antigenic tag. Antibodies raised against the polypeptide of the present invention can be used to indirectly detect the second protein by binding to the polypeptide. Moreover, because secreted proteins target cellular locations based on trafficking signals, the polypeptides of the present invention can be used as targeting molecules once fused to other proteins.

Examples of domains that can be fused to polypeptides of the present invention include not only heterologous signal sequences, but also other heterologous functional regions. The fusion does not necessarily need to be direct, but may occur through linker sequences.

Moreover, fusion proteins may also be engineered to improve characteristics of the polypeptide of the present invention. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence during purification from the host cell or subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to facilitate handling of polypeptides are familiar and routine techniques in the art.

Moreover, polypeptides of the present invention, including fragments, and specifically epitopes, can be combined with parts of the constant domain of immunoglobulins (IgG), as described above, resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life in vivo. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP A 394,827; Traunecker et al., *Nature*, 331:84-86 (1988).) Fusion proteins having disulfide-linked dimeric structures (due to the IgG) can also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis et al., *J. Biochem.*, 270:3958-3964 (1995)).

Similarly, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP-A 0232 262.) Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, Bennett et al., *J. Molecular Recognition*, 8:52-58 (1995); Johanson et al., *J. Biol. Chem.*, 270:9459-9471 (1995)).

Moreover, the polypeptides of the present invention can be fused to marker sequences, such as a peptide which facilitates purification of the fused polypeptide. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, 5 Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz et al., *Proc. Natl. Acad. Sci. USA*, 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Another peptide tag useful for purification, the "HA" tag, corresponds to an epitope derived from the influenza hemagglutinin protein. (Wilson et al., *Cell*, 37:767 10 (1984).)

Thus, any of these above fusions can be engineered using the polynucleotides or the polypeptides of the present invention.

#### **Vectors, Host Cells, and Protein Production**

15 The present invention also relates to vectors containing the polynucleotide of the present invention, host cells, and the production of polypeptides by recombinant techniques. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host 20 cells.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line 25 and then transduced into host cells.

The polynucleotide insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp, phoA and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The 30 expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding

portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one  
5 selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells;  
10 insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, 293, and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from QIAGEN, Inc.; pBluescript vectors, Phagescript vectors, pNH8A,  
15 pNH16a, pNH18A, pNH46A, available from Stratagene Cloning Systems, Inc.; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia Biotech, Inc. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

20 Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., *Basic Methods In Molecular Biology* (1986). It is specifically contemplated that the  
25 polypeptides of the present invention may in fact be expressed by a host cell lacking a recombinant vector.

A polypeptide of this invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography,  
30 phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most

preferably, high performance liquid chromatography ("HPLC") is employed for purification.

Polypeptides of the present invention can also be recovered from: products purified from natural sources, including bodily fluids, tissues and cells, whether  
5 directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect, and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition,  
10 polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Thus, it is well known in the art that the N-terminal methionine encoded by the translation initiation codon generally is removed with high efficiency from any protein after translation in all eukaryotic cells. While the N-terminal methionine on most proteins also is efficiently removed  
15 in most prokaryotes, for some proteins, this prokaryotic removal process is inefficient, depending on the nature of the amino acid to which the N-terminal methionine is covalently linked. In addition, a methionine codon may be appropriately added to vectors of the present invention, for the proper translation of polypeptides of the present invention which lack an N-terminal methionine.

20 In addition to encompassing host cells containing the vector constructs discussed herein, the invention also encompasses primary, secondary, and immortalized host cells of vertebrate origin, particularly mammalian origin, that have been engineered to delete or replace endogenous genetic material (e.g., BMP coding sequence), and/or to include genetic material (e.g., heterologous polynucleotide  
25 sequences) that is operably associated with BMP polynucleotides of the invention, and which activates, alters, and/or amplifies endogenous BMP polynucleotides. For example, techniques known in the art may be used to operably associate heterologous control regions (e.g., promoter and/or enhancer) and endogenous BMP polynucleotide sequences via homologous recombination (see, e.g., U.S. Patent NO: 5,641,670,  
30 issued June 24, 1997; International Publication NO: WO 96/29411, published September 26, 1996; International Publication NO: WO 94/12650, published August

4, 1994; Koller et al., *Proc. Natl. Acad. Sci. USA*, 86:8932-8935 (1989); and Zijlstra et al., *Nature*, 342:435-438 (1989), the disclosures of each of which are incorporated by reference in their entireties).

In addition, polypeptides of the invention can be chemically synthesized using techniques known in the art (e.g., see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., N.Y., and Hunkapiller et al., *Nature*, 310:105-111 (1984)). For example, a peptide corresponding to a fragment of the BMP polypeptides of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the BMP polynucleotide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino acids, designer amino acids such as  $\beta$ -methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Additionally, methods for production of numerous members of the TGF- $\beta$  superfamily useful in making the polypeptides of the present invention are known and/or described in the literature. For example, the structure and methods for production of many BMPs, including BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 and BMP-7, are disclosed, for instance, in U.S. Pat. Nos. 5,108,922; 5,013,649; 5,116,738; 5,106,748; 5,187,076; and 5,141,905; BMP-8, disclosed in PCT publication WO91/18098; BMP-9, disclosed in PCT publication WO93/00432; BMP-10, disclosed in PCT application WO94/26893; BMP-11, disclosed in PCT application WO94/26892; BMP-12 and BMP-13, disclosed in PCT application WO 95/16035. The structure of Vgr-2, and the growth and differentiation factors (GDFs), including those described in PCT applications WO94/15965; WO94/15949; WO95/01801; WO95/01802; WO94/21681; WO94/15966; and others are also

known. Other TGF-beta proteins which may be useful in the present invention include BIP, disclosed in WO94/01557; and MP52, disclosed in PCT application WO93/16099. Methods for production of heterodimeric proteins comprising two distinct monomeric units, each comprising the amino acid sequence of one of the above TGF-beta proteins, are described in WO93/09229. The disclosures of all of the above applications are hereby incorporated by reference.

The invention encompasses BMP polypeptides which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH<sub>4</sub>; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of procaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Also provided by the invention are chemically modified derivatives of BMP which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U.S. Patent NO: 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at



predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between  
5 about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or  
10 lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those  
15 skilled in the art, e.g., EP 0 401 384, herein incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol. 20:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated  
20 polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for  
25 therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight,  
30 branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be

performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules.

- 5 Selective proteins chemically modified at the N-terminus modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl  
10 group containing polymer is achieved.

- The BMP polypeptides of the invention may be in monomers or multimers (i.e., dimers, trimers, tetramers and higher multimers). Accordingly, the present invention relates to monomers and multimers of the BMP polypeptides of the invention, their preparation, and compositions (preferably, pharmaceutical  
15 compositions) containing them. In specific embodiments, the polypeptides of the invention are monomers, dimers, trimers or tetramers. In additional embodiments, the multimers of the invention are at least dimers, at least trimers, or at least tetramers.

- Multimers encompassed by the invention may be homomers or heteromers. As used herein, the term homomer, refers to a multimer containing only BMP  
20 polypeptides of the invention (including BMP fragments, variants, splice variants, and fusion proteins, as described herein). These homomers may contain BMP polypeptides having identical or different amino acid sequences. In a specific embodiment, a homomer of the invention is a multimer containing only BMP polypeptides having an identical amino acid sequence. In another specific  
25 embodiment, a homomer of the invention is a multimer containing BMP polypeptides having different amino acid sequences. In specific embodiments, the multimer of the invention is a homodimer (*e.g.*, containing BMP polypeptides having identical or different amino acid sequences) or a homotrimer (*e.g.*, containing BMP polypeptides having identical and/or different amino acid sequences). In additional embodiments,  
30 the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

As used herein, the term heteromer refers to a multimer containing one or more heterologous polypeptides (*i.e.*, polypeptides of different proteins) in addition to the BMP polypeptides of the invention. In a specific embodiment, the multimer of the invention is a heterodimer, a heterotrimer, or a heterotetramer. In additional  
5   embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

Multimers of the invention may be the result of hydrophobic, hydrophilic, ionic and/or covalent associations and/or may be indirectly linked, by for example, liposome formation. Thus, in one embodiment, multimers of the invention, such as,  
10   for example, homodimers or homotrimers, are formed when polypeptides of the invention contact one another in solution. In another embodiment, heteromultimers of the invention, such as, for example, heterotrimers or heterotetramers, are formed when polypeptides of the invention contact antibodies to the polypeptides of the invention (including antibodies to the heterologous polypeptide sequence in a fusion  
15   protein of the invention) in solution. In other embodiments, multimers of the invention are formed by covalent associations with and/or between the BMP polypeptides of the invention. Such covalent associations may involve one or more amino acid residues contained in the polypeptide sequence ( *e.g.*, that recited in the sequence listing, or contained in the polypeptide encoded by the clone BMP). In one  
20   instance, the covalent associations are cross-linking between cysteine residues located within the polypeptide sequences which interact in the native (*i.e.*, naturally occurring) polypeptide. In another instance, the covalent associations are the consequence of chemical or recombinant manipulation. Alternatively, such covalent associations may involve one or more amino acid residues contained in the  
25   heterologous polypeptide sequence in a BMP fusion protein. In one example, covalent associations are between the heterologous sequence contained in a fusion protein of the invention (see, *e.g.*, US Patent Number 5,478,925). In a specific example, the covalent associations are between the heterologous sequence contained in a BMP-Fc fusion protein of the invention (as described herein). In another specific  
30   example, covalent associations of fusion proteins of the invention are between heterologous polypeptide sequence from another TNF family ligand/receptor member

that is capable of forming covalently associated multimers, such as for example, osteoprotegerin (see, e.g., International Publication NO: WO 98/49305, the contents of which are herein incorporated by reference in its entirety).

The multimers of the invention may be generated using chemical techniques known in the art. For example, polypeptides desired to be contained in the multimers of the invention may be chemically cross-linked using linker molecules and linker molecule length optimization techniques known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, multimers of the invention may be generated using techniques known in the art to form one or more inter-molecule cross-links between the cysteine residues located within the sequence of the polypeptides desired to be contained in the multimer (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Further, polypeptides of the invention may be routinely modified by the addition of cysteine or biotin to the C terminus or N-terminus of the polypeptide and techniques known in the art may be applied to generate multimers containing one or more of these modified polypeptides (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, techniques known in the art may be applied to generate liposomes containing the polypeptide components desired to be contained in the multimer of the invention (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

Alternatively, multimers of the invention may be generated using genetic engineering techniques known in the art. In one embodiment, polypeptides contained in multimers of the invention are produced recombinantly using fusion protein technology described herein or otherwise known in the art (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In a specific embodiment, polynucleotides coding for a homodimer of the invention are generated by ligating a polynucleotide sequence encoding a polypeptide of the invention to a sequence encoding a linker polypeptide and then further to a synthetic polynucleotide encoding the translated product of the polypeptide in the reverse orientation from the original C-terminus to the N-terminus (lacking the leader

sequence) (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In another embodiment, recombinant techniques described herein or otherwise known in the art are applied to generate recombinant polypeptides of the invention which contain a transmembrane domain (or hydrophobic or signal  
5 peptide) and which can be incorporated by membrane reconstitution techniques into liposomes (see, e.g., US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

### Uses of the Polynucleotides

10 Each of the polynucleotides identified herein can be used in numerous ways as reagents. The following description should be considered exemplary and utilizes known techniques.

The polynucleotides of the present invention are useful for chromosome identification. There exists an ongoing need to identify new chromosome markers,  
15 since few chromosome marking reagents, based on actual sequence data (repeat polymorphisms), are presently available. Each polynucleotide of the present invention can be used as a chromosome marker.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the sequences shown in SEQ ID NO:X. Primers can be  
20 selected using computer analysis so that primers do not span more than one predicted exon in the genomic DNA. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the SEQ ID NO:X will yield an amplified fragment.

25 Similarly, somatic hybrids provide a rapid method of PCR mapping the polynucleotides to particular chromosomes. Three or more clones can be assigned per day using a single thermal cycler. Moreover, sublocalization of the polynucleotides can be achieved with panels of specific chromosome fragments. Other gene mapping strategies that can be used include in situ hybridization,  
30 prescreening with labeled flow-sorted chromosomes, and preselection by hybridization to construct chromosome specific-cDNA libraries.

Precise chromosomal location of the polynucleotides can also be achieved using fluorescence in situ hybridization (FISH) of a metaphase chromosomal spread. This technique uses polynucleotides as short as 500 or 600 bases; however, polynucleotides 2,000-4,000 bp are preferred. For a review of this technique, see  
5 Verma et al., "Human Chromosomes: a Manual of Basic Techniques," Pergamon Press, New York (1988).

For chromosome mapping, the polynucleotides can be used individually (to mark a single chromosome or a single site on that chromosome) or in panels (for marking multiple sites and/or multiple chromosomes). Preferred polynucleotides  
10 correspond to the noncoding regions of the cDNAs because the coding sequences are more likely conserved within gene families, thus increasing the chance of cross hybridization during chromosomal mapping.

Once a polynucleotide has been mapped to a precise chromosomal location, the physical position of the polynucleotide can be used in linkage analysis. Linkage  
15 analysis establishes coinheritance between a chromosomal location and presentation of a particular disease. (Disease mapping data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library) .) Assuming 1 megabase mapping resolution and one gene per 20 kb, a cDNA precisely localized to a chromosomal region associated  
20 with the disease could be one of 50-500 potential causative genes.

Thus, once coinheritance is established, differences in the polynucleotide and the corresponding gene between affected and unaffected individuals can be examined. First, visible structural alterations in the chromosomes, such as deletions or translocations, are examined in chromosome spreads or by PCR. If no structural  
25 alterations exist, the presence of point mutations are ascertained. Mutations observed in some or all affected individuals, but not in normal individuals, indicates that the mutation may cause the disease. However, complete sequencing of the polypeptide and the corresponding gene from several normal individuals is required to distinguish the mutation from a polymorphism. If a new polymorphism is identified, this  
30 polymorphic polypeptide can be used for further linkage analysis.

Furthermore, increased or decreased expression of the gene in affected individuals as compared to unaffected individuals can be assessed using polynucleotides of the present invention. Any of these alterations (altered expression, chromosomal rearrangement, or mutation) can be used as a diagnostic or prognostic  
5 marker.

In addition to the foregoing, a polynucleotide can be used to control gene expression through triple helix formation or antisense DNA or RNA. Both methods rely on binding of the polynucleotide to DNA or RNA. For these techniques, preferred polynucleotides are usually 20 to 40 bases in length and complementary to  
10 either the region of the gene involved in transcription (triple helix - see Lee et al., *Nucl. Acids Res.*, 6:3073 (1979); Cooney et al., *Science*, 241:456 (1988); and Dervan et al., *Science*, 251:1360 (1991)) or to the mRNA itself (antisense - Okano, *J. Neurochem.*, 56:560 (1991); Oligodeoxy-nucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988).) Triple helix formation optimally  
15 results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques are effective in model systems, and the information disclosed herein can be used to design antisense or triple helix polynucleotides in an effort to treat disease.

Polynucleotides of the present invention are also useful in gene therapy. One  
20 goal of gene therapy is to insert a normal gene into an organism having a defective gene, in an effort to correct the genetic defect. The polynucleotides disclosed in the present invention offer a means of targeting such genetic defects in a highly accurate manner. Another goal is to insert a new gene that was not present in the host genome, thereby producing a new trait in the host cell.

The polynucleotides are also useful for identifying individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for  
25 identifying personnel. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification  
30

difficult. The polynucleotides of the present invention can be used as additional DNA markers for RFLP.

The polynucleotides of the present invention can also be used as an alternative to RFLP, by determining the actual base-by-base DNA sequence of selected portions of an individual's genome. These sequences can be used to prepare PCR primers for amplifying and isolating such selected DNA, which can then be sequenced. Using this technique, individuals can be identified because each individual will have a unique set of DNA sequences. Once an unique ID database is established for an individual, positive identification of that individual, living or dead, can be made from extremely small tissue samples.

Forensic biology also benefits from using DNA-based identification techniques as disclosed herein. DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, semen, etc., can be amplified using PCR. In one prior art technique, gene sequences amplified from polymorphic loci, such as DQa class II HLA gene, are used in forensic biology to identify individuals. (Erlich, H., PCR Technology, Freeman and Co. (1992).) Once these specific polymorphic loci are amplified, they are digested with one or more restriction enzymes, yielding an identifying set of bands on a Southern blot probed with DNA corresponding to the DQa class II HLA gene. Similarly, polynucleotides of the present invention can be used as polymorphic markers for forensic purposes.

There is also a need for reagents capable of identifying the source of a particular tissue. Such need arises, for example, in forensics when presented with tissue of unknown origin. Appropriate reagents can comprise, for example, DNA probes or primers specific to particular tissue prepared from the sequences of the present invention. Panels of such reagents can identify tissue by species and/or by organ type. In a similar fashion, these reagents can be used to screen tissue cultures for contamination.

In the very least, the polynucleotides of the present invention can be used as molecular weight markers on Southern gels, as detection and diagnostic probes for the presence of a specific mRNA in a particular cell type, as a probe to "subtract-out"



known sequences in the process of discovering novel polynucleotides, for selecting and making oligomers for attachment to a "gene chip" or other support, to raise anti-DNA antibodies using DNA immunization techniques, and as an antigen to elicit an immune response.

5

### Uses of the Polypeptides

Each of the polypeptides identified herein can be used in numerous ways. The following description should be considered exemplary and utilizes known techniques.

A polypeptide of the present invention can be used to assay protein levels in a biological sample using antibody-based techniques. For example, protein expression  
10 in tissues can be studied with classical immunohistological methods. (Jalkanen et al., *J. Cell. Biol.*, 101:976-985 (1985); Jalkanen et al., *J. Cell. Biol.*, 105:3087-3096 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and  
15 the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine (125I, 121I), carbon (14C), sulfur (35S), tritium (3H), indium (112In), and technetium (99mTc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

In addition to assaying secreted protein levels in a biological sample, proteins  
20 can also be detected *in vivo* by imaging. Antibody labels or markers for *in vivo* imaging of protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as  
25 deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma.

A protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example, 131I, 112In, 99mTc), a radio-opaque substance, or a material detectable by nuclear  
30 magnetic resonance, is introduced (for example, parenterally, subcutaneously, or intraperitoneally) into the mammal. It will be understood in the art that the size of the

subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of <sup>99m</sup>Tc. The labeled antibody or antibody fragment will then  
5 preferentially accumulate at the location of cells which contain the specific protein. In vivo tumor imaging is described in S.W. Burchiel et al., "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments." (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982)).

10 Thus, the invention provides a detection or diagnostic method of a disorder, which involves (a) assaying the expression of a polypeptide of the present invention in cells or body fluid of an individual; (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is  
15 indicative of a marker for a cell type, cell condition, or disorder.

Moreover, polypeptides of the present invention can be used to treat disease. For example, patients can be administered a polypeptide of the present invention in an effort to replace absent or decreased levels of the polypeptide (e.g., insulin), to supplement absent or decreased levels of a different polypeptide (e.g., hemoglobin S  
20 for hemoglobin B), to inhibit the activity of a polypeptide (e.g., an oncogene), to activate the activity of a polypeptide (e.g., by binding to a receptor), to reduce the activity of a membrane bound receptor by absorbing free ligand (e.g., soluble TNF receptors used in reducing inflammation), or to bring about a desired response (e.g., blood vessel growth).

25 Similarly, antibodies directed to a polypeptide of the present invention can also be used to treat disease. For example, administration of an antibody directed to a polypeptide of the present invention can bind and reduce levels of the polypeptide. Similarly, administration of an antibody can activate the polypeptide, such as by binding to a polypeptide bound to a membrane (receptor).

30 At the very least, the polypeptides of the present invention can be used as molecular weight markers on SDS-PAGE gels or on molecular sieve gel filtration

columns using methods well known to those of skill in the art. Polypeptides can also be used to raise antibodies, which in turn are used to measure protein expression from a recombinant cell, as a way of assessing transformation of the host cell. Moreover, the polypeptides of the present invention can be used to test the following biological activities.

#### **Treatment of Osteoarthritis, Cartilage Defects and Tissue Repair**

The methods and compositions of the present invention may comprise administration of a BMP to a patient or site in need of cartilage repair, formation or maintenance. For sequential administration, the active agent may be encapsulated or otherwise maintained in contact with a carrier which provides for slow release of the agent.

The compositions of the invention may comprise, in addition to a BMP, other therapeutically useful agents including growth factors such as parathyroid hormone-related peptide, epidermal growth factor (EGF), transforming growth factor- $\alpha$ , activins, inhibins, platelet derived growth factor (PDGF), fibroblast growth factor-1 through 17 (preferred FGFs are bFGF and FGF-4), and fibroblast growth factor-4 (FGF-4), parathyroid hormone (PTH), leukemia inhibitory factor (LIF/HILDA/DIA), and insulin-like growth factors (IGF-I and IGF-II). Portions of these agents may also be used in compositions of the present invention. The compositions may also include an appropriate matrix for instance, for supporting the composition and providing a surface for cartilage or for other connective tissue growth. The matrix may provide slow release of the protein and/or the appropriate environment for presentation thereof.

The methods and compositions of the present invention employ proteins which are able to induce cartilaginous tissue, bone, or other tissue formation in circumstances where such tissue is not normally formed, and has application in the healing of cartilage, for example articular cartilage tears, deformities and other cartilage defects in humans and other animals. Such methods and compositions employing cartilaginous tissue inducing proteins may have prophylactic use in preventing damage to cartilaginous tissue, as well as use in the improved fixation of

cartilage to bone or other tissues, and in repairing defects to cartilage tissue. De novo cartilaginous tissue formation induced by a composition of the present invention contributes to the repair of congenital, trauma induced, or other cartilage defects of other origin, and is also useful in surgery for attachment or repair of cartilage. The methods and compositions of the invention may also be useful in the treatment of arthritis and other cartilage defects. The methods and compositions of the present invention can also be used in other indications wherein it is desirable to heal or regenerate cartilage and bone tissue. Such indications include, without limitation, regeneration or repair of injuries to the articular cartilage. The methods and compositions of the present invention may provide an environment to attract cartilage-forming cells, stimulate growth of cartilage-forming cells or induce differentiation of progenitors of cartilage-forming cells and chondrocytes.

The compositions and methods of the present invention may also be useful for treating cell populations, such as embryonic cells or stem cell populations, to enhance or enrich the growth, survival and/or differentiation of the cells into chondrocytes or other cell types. In another embodiment, the compositions and methods of the present invention may be used to treat chondrocytic cell lines, such as articular chondrocytes, in order to maintain chondrocytic phenotype and survival of the cells. The treated cell populations may be useful for gene therapy applications. (See *infra.*).

The proteins useful in the methods of the present invention are useful for inducing the formation, maintenance and survival of chondrocytes and/or cartilaginous or bone tissue. It is contemplated that these proteins may have the ability to induce the formation of other types of tissue, such as tendon and ligament, as well. The cartilaginous tissue-inducing methods and compositions provided herein also may include factors encoded by the sequences similar to those of naturally-occurring BMPs, into which modifications are naturally provided (e.g. allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of BMPs. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with cartilaginous tissue growth factor polypeptides of

naturally-occurring BMPs may possess cartilaginous, bone, or other tissue growth factor biological properties in common therewith. Thus, they may be employed as biologically active substitutes for naturally-occurring cartilaginous tissue inducing polypeptides in therapeutic methods and compositions.

- 5           The therapeutic method includes administering the composition topically, systemically, or locally as an injectable and/or implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of tissue damage.
- 10   Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than the proteins which may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the composition in the methods of the invention. In addition, the compositions of the present invention may be used in
- 15   conjunction with presently available treatments for cartilage and bone injuries, such as suture (e.g., vicryl sutures or surgical gut sutures, Ethicon Inc., Somerville, N.J.) or cartilage or bone allograft or autograft, in order to enhance or accelerate the healing potential of the suture or graft. For example, the suture, allograft or autograft may be soaked in the compositions of the present invention prior to implantation. It may also
- 20   be possible to incorporate the protein or composition of the invention onto suture materials, for example, by freeze-drying.

- The compositions of the present invention may include an appropriate matrix and/or sequestering agent as a carrier. For instance, the matrix may support the composition or provide a surface for cartilaginous or bone tissue formation and/or
- 25   other tissue formation. The matrix may provide slow release of the protein and/or the appropriate environment for presentation thereof. The sequestering agent may be a substance which aids in ease of administration through injection or other means, or may slow the migration of protein from the site of application.

- Examples of sustained release carriers include semipermeable polymer
- 30   matrices in the form of shaped articles such as suppositories or capsules. Implantable or microcapsular sustained release matrices include polylactides (U.S. Patent No.

3,773,319; EP 58,481), copolymers of L-glutamic acid and ethyl-L-glutamate (Sidman et al., Biopolymers 22: 547-56 (1985)); poly(2-hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al., J. Biomed. Mater. Res., 15:167-277 (1981); Langer, Chem. Tech., 12:98-105 (1982)) (the above references are incorporated  
5 herein in their entirety). The choice of a carrier material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined. Further matrices are comprised of pure proteins or extracellular  
10 matrix components. Other potential matrices are nonbiodegradable and chemically defined. Preferred matrices include collagen-based materials, including sponges, such as Helistat' (Integra LifeSciences, Plainsboro, N.J.), or collagen in an injectable form, as well as sequestering agents, which may be biodegradable, for example hyaluronic acid derived. Biodegradable materials, such as cellulose films, or surgical meshes,  
15 may also serve as matrices. Such materials could be sutured into an injury site, or wrapped around the cartilage.

Another preferred class of carrier are polymeric matrices, including polymers of poly(lactic acid), poly(glycolic acid) and copolymers of lactic acid and glycolic acid. These matrices may be in the form of a sponge, or in the form of porous  
20 particles, and may also include a sequestering agent. Suitable polymer matrices are described, for example, in WO93/00050, the disclosure of which is incorporated herein by reference. For morphogenic devices comprising a biocompatible matrix made up of particles or porous materials, the pores are preferably of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation.  
25 Various matrices known in the art can be employed (see, e.g., U.S. Pat. Nos. 4,975,526; 5,162,114; 5,171,574 and WO 91/18558, which are herein incorporated by reference).

The particle size should be within the range of 70  $\mu\text{m}$  to 850  $\mu\text{m}$ , preferably 70  $\mu\text{m}$  to 420  $\mu\text{m}$ , most preferably 150  $\mu\text{m}$  to 420  $\mu\text{m}$ . The matrix may be fabricated  
30 by close packing particulate material into a shape spanning the particular tissue defect to be treated. Alternatively, a material that is biocompatible, and preferably

biodegradable in vivo may be structured to serve as a temporary scaffold and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation.

Useful matrix materials comprise, for example, collagen; homopolymers or  
5 copolymers of glycolic acid, lactic acid, and butyric acid, including derivatives thereof; and ceramics, such as hydroxyapatite, tricalcium phosphate and other calcium phosphates. Various combinations of these or other suitable matrix materials also may be useful as determined by the assays set forth herein.

Preferred families of sequestering agents include blood, fibrin clot and/or  
10 cellulosic materials such as alkylcelluloses (including hydroxyalkylcelluloses), including methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropyl-methylcellulose, and carboxymethylcellulose, the most preferred being cationic salts of carboxymethylcellulose (CMC). Other preferred sequestering agents include  
15 hyaluronic acid, sodium alginate, poly(ethylene glycol), polyoxyethylene oxide, carboxyvinyl polymer and poly(vinyl alcohol). The amount of sequestering agent useful herein is 0.5-20 wt %, preferably 1-10 wt % based on total formulation weight, which represents the amount necessary to prevent desorption of the protein from the polymer matrix and to provide appropriate handling of the composition, yet not so  
20 much that the progenitor cells are prevented from infiltrating the matrix, thereby providing the protein the opportunity to assist the activity of the progenitor cells.

Currently preferred carriers include particulate, demineralized, guanidine-extracted, species-specific (allogenic) bone, and specially treated particulate, protein-extracted, demineralized xenogenic bone. Optionally, such xenogenic bone powder  
25 matrices also may be treated with proteases such as trypsin. Preferably, the xenogenic matrices are treated with one or more fibril modifying agents to increase the intraparticle intrusion volume (porosity) and surface area. Useful modifying agents include solvents such as dichloromethane, trichloroacetic acid, acetonitrile and acids such as trifluoroacetic acid and hydrogen fluoride. The currently preferred fibril-  
30 modifying agent useful in formulating the matrices of this invention is a heated aqueous medium, preferably an acidic aqueous medium having a pH less than about

pH 4.5, most preferably having a pH within the range of about pH 2-pH 4. A currently preferred heated acidic aqueous medium is 0.1% acetic acid which has a pH of about 3. Heating demineralized, delipidated, guanidine-extracted bone collagen in an aqueous medium at elevated temperatures (e.g., in the range of about 37°C to 5 65°C, preferably in the range of about 45°C to 60°C) for approximately one hour generally is sufficient to achieve the desired surface morphology. Although the mechanism is not clear, it is hypothesized that the heat treatment alters the collagen fibrils, resulting in an increase in the particle surface area.

Demineralized guanidine-extracted xenogenic bovine bone comprises a 10 mixture of additional materials that may be fractionated further using standard biomolecular purification techniques. For example, chromatographic separation of extract components followed by addition back to active matrix of the various extract fractions corresponding to the chromatogram peaks may be used to improve matrix properties by fractionating away inhibitors of bone or tissue-inductive activity.

15 The matrix may also be substantially depleted in residual heavy metals. Treated as disclosed herein, individual heavy metal concentrations in the matrix can be reduced to less than about 1 ppm.

The currently preferred carrier material is a xenogenic bone-derived particulate matrix treated as described herein. This carrier may be replaced by either a 20 biodegradable-synthetic or a synthetic-inorganic matrix (e.g., hydroxyapatite (HAP), collagen, carboxymethylcellulose, tricalcium phosphate or polylactic acid, polyglycolic acid, polybutyric acid and various copolymers thereof.)

Matrix geometry, particle size, the presence of surface charge, and the degree of both intra- and inter-particle porosity are all important to successful matrix 25 performance. Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and morphogenic proteins all play a role in achieving successful tissue induction.

A successful carrier for BMPs should perform several important functions. It should act as a slow release delivery system of BMP, protect the BMP from non- 30 specific proteolysis, and should accommodate each step of the cellular responses involved in progenitor cell induction during tissue development.



In addition, selected materials must be biocompatible in vivo and preferably biodegradable; the carrier preferably acts as a temporary scaffold until replaced completely by new bone or tissue. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates in vivo. In bones, the  
5 dissolution rates can vary according to whether the implant is placed in cortical or trabecular bone.

The preferred osteogenic device matrix material, prepared from xenogenic bone and treated as disclosed herein, produces an implantable material useful in a variety of clinical settings. In addition to its use as a matrix for bone formation in  
10 various orthopedic, periodontal, and reconstructive procedures, the matrix also may be used as a sustained release carrier, or as a collagenous coating for orthopedic or general prosthetic implants.

The matrix may be shaped as desired in anticipation of surgery or shaped by the physician or technician during surgery. It is preferred to shape the matrix to span a  
15 tissue defect and to take the desired form of the new tissue. In the case of bone repair of a non-union defect, for example, it is desirable to use dimensions that span the non-union. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted. Thus, the material may be used for topical, subcutaneous, intraperitoneal, or intramuscular implants. In bone formation  
20 procedures, the material is slowly absorbed by the body and is replaced by bone in the shape of or very nearly the shape of the implant.

The matrix may comprise a shape-retaining solid made of loosely-adhered particulate material, e.g., collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue.  
25 Masticated muscle or other tissue may also be used. Large allogenic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and packed with particles comprising dispersed BMPs. The matrix may also take the form of a paste or a hydrogel.

When the carrier material comprises a hydrogel matrix, it refers to a three  
30 dimensional network of cross-linked hydrophilic polymers in the form of a gel substantially composed of water, preferably but not limited to gels being greater than

90% water. Hydrogel matrices can carry a net positive or net negative charge, or may be neutral. A typical net negative charged matrix is alginate. Hydrogels carrying a net positive charge may be typified by extracellular matrix components such as collagen and laminin. Examples of commercially available extracellular matrix components  
5 include Matrigel™ and Vitrogen™. An example of a net neutral hydrogel is highly crosslinked polyethylene oxide, or polyvinylalcohol.

Various growth factors, cytokines, hormones, trophic agents and therapeutic compositions including antibiotics and chemotherapeutic agents, enzymes, enzyme inhibitors and other bioactive agents also may be adsorbed onto or dispersed within  
10 the carrier material comprising the BMP, and will also be released over time at the implantation site as the matrix material is slowly absorbed.

In addition to the naturally-derived bone matrices described above, useful matrices may also be formulated synthetically by adding together reagents that have been appropriately modified. One example of such a matrix is the porous,  
15 biocompatible, in vivo biodegradable synthetic matrix disclosed in WO91/18558, the disclosure of which is hereby incorporated by reference.

Briefly, the matrix comprises a porous crosslinked structural polymer of biocompatible, biodegradable collagen, most preferably tissue-specific collagen, and appropriate, tissue-specific glycosaminoglycans as tissue-specific cell attachment  
20 factors. Bone tissue-specific collagen (e.g., Type I collagen) derived from a number of sources may be suitable for use in these synthetic matrices, including soluble collagen, acid-soluble collagen, collagen soluble in neutral or basic aqueous solutions, as well as those collagens which are commercially available. In addition, Type II collagen, as found in cartilage, also may be used in combination with Type I collagen.

25 Glycosaminoglycans (GAGs) or mucopolysaccharides are polysaccharides made up of residues of hexoamines glycosidically bound and alternating in a more-or-less regular manner with either hexouronic acid or hexose moieties. GAGs are of animal origin and have a tissue specific distribution (see, e.g., Dodgson et al., in Carbohydrate Metabolism and its Disorders, Dickens et al., eds., Vol. 1, Academic  
30 Press (1968)). Reaction with the GAGs also provides collagen with another valuable

property, i.e., inability to provoke an immune reaction (foreign body reaction) from an animal host.

Useful GAGs include those containing sulfate groups, such as hyaluronic acid, heparin, heparin sulfate, chondroitin 6-sulfate, chondroitin 4-sulfate, dermatan sulfate, and keratin sulfate. For osteogenic devices, chondroitin 6-sulfate currently is preferred. Other GAGs also may be suitable for forming the matrix described herein, and those skilled in the art will either know or be able to ascertain other suitable GAGs using no more than routine experimentation. For a more detailed description of mucopolysaccharides, see Aspinall, *Polysaccharides*, Pergamon Press, Oxford (1970). Collagen can be reacted with a GAG in aqueous acidic solutions, preferably in diluted acetic acid solutions. By adding the GAG dropwise into the aqueous collagen dispersion, coprecipitates of tangled collagen fibrils coated with GAG results. This tangled mass of fibers then can be homogenized to form a homogeneous dispersion of fine fibers and then filtered and dried.

Insolubility of the collagen-GAG products can be raised to the desired degree by covalently cross-linking these materials, which also serves to raise the resistance to resorption of these materials. In general, any covalent G60 cross-linking method suitable for cross-linking collagen also is suitable for cross-linking these composite materials, although cross-linking by a dehydrothermal process is preferred.

When dry, the cross-linked particles are essentially spherical with diameters of about 500  $\mu\text{m}$ . Scanning electron microscopy shows pores of about 20  $\mu\text{m}$  on the surface and 40  $\mu\text{m}$  on the interior. The interior is made up of both fibrous and sheet-like structures, providing surfaces for cell attachment. The voids interconnect, providing access to the cells throughout the interior of the particle. The material appears to be roughly 99.59% void volume, making the material very efficient in terms of the potential cell mass that can be grown per gram of microcarrier.

Another useful synthetic matrix is one formulated from biocompatible, in vivo biodegradable synthetic polymers, such as those composed of glycolic acid, lactic acid and/or butyric acid, including copolymers and derivatives thereof. These polymers are well described in the art and are available commercially. For example, polymers composed of polylactic acid (e.g., MW 100 kDa), 80% polylactide/20%

glycoside or poly 3-hydroxybutyric acid (e.g., MW 30 kDa) all may be purchased from PolySciences, Inc. The polymer compositions generally are obtained in particulate form and the morphogenic devices preferably fabricated under nonaqueous conditions (e.g., in an ethanol-trifluoroacetic acid solution, EtOH/TFA) to avoid

5 hydrolysis of the polymers. In addition, one can alter the morphology of the particulate polymer compositions, for example to increase porosity, using any of a number of particular solvent treatments known in the art.

In another embodiment of this invention, an implantable prosthetic device comprising a BMP is provided. Any prosthetic implant selected for a particular

10 treatment by the skilled practitioner may be used in combination with a composition comprising at least one BMP according to this invention. The prosthesis may be made from a material comprising metal or ceramic. Preferred prosthetic devices are selected from the group consisting of a hip device, a screw, a rod and a titanium cage for spine fusion.

15 The BMP composition is disposed on the prosthetic implant on a surface region that is implantable adjacent to a target tissue in the mammal. Preferably, the mammal is a human patient. The composition is disposed on the surface of the implant in an amount sufficient to promote enhanced tissue growth into the surface. The amount of the composition sufficient to promote enhanced tissue growth may be

20 determined empirically by those of skill in the art using bioassays such as those described herein and in Rueger et al., U.S. Pat. No. 5,344,654, which is incorporated herein by reference. Preferably, animal studies are performed to optimize the concentration of the composition components before a similar prosthetic device is used in the human patient. Such prosthetic devices will be useful for repairing

25 orthopedic defects, injuries or anomalies in the treated mammal.

Thus this invention also provides a method for promoting in vivo integration of an implantable prosthetic device into a target tissue of a mammal comprising the steps of providing on a surface of the prosthetic device a composition comprising at least one BMP, and implanting the device in a mammal at a locus where the target

tissue and the surface of the prosthetic device are maintained at least partially in contact for a time sufficient to permit enhanced tissue growth between the target tissue and the device.

Additional optional components useful in the practice of the subject application include, e.g. cryogenic protectors such as mannitol, sucrose, lactose, glucose, or glycine (to protect the protein from degradation during lyophilization), antimicrobial preservatives such as methyl and propyl parabens and benzyl alcohol; antioxidants such as EDTA, citrate and BHT (butylated hydroxytoluene); and surfactants such as poly(sorbates) and poly(oxyethylenes); etc.

As described above, the compositions of the invention may be employed in methods for treating a number of cartilage and bone defects, such as the regeneration of cartilaginous tissue or bone tissue in areas of cartilage and bone damage, to assist in repair of tears of cartilage tissue and various other types of tissue defects or wounds. These methods, according to the invention, entail administering to a patient needing such cartilaginous tissue, bone tissue, or other tissue repair, a composition comprising an effective amount of a BMP alone or in combination with additional therapeutic agents.

A further aspect of the invention is a therapeutic method and composition for inducing or maintaining chondrocytes or cartilaginous tissue, for repairing cartilaginous tissue and bone, for repairing cartilage and bone as well as treating arthritis and other conditions related to arthritis, cartilage, and bone defects. Such compositions comprise a therapeutically effective amount of one or more BMPs of the present invention, in admixture with a pharmaceutically acceptable vehicle, carrier or matrix.

Thus, the morphogenic compositions and devices comprising a BMP disclosed herein will permit the physician to treat a variety of tissue injuries, tissue degenerative or disease conditions and disorders that can be ameliorated or remedied by localized, stimulated tissue regeneration or repair.

The morphogenic devices of this invention may be used to induce local tissue formation from a progenitor cell in a mammal by implanting the device at a locus accessible to at least one progenitor cell of the mammal. The morphogenic devices of

this invention may be used alone or in combination with other therapies for tissue repair and regeneration.

Certain BMPs which are known to be osteogenic can also induce neuronal cell differentiation. Embryonic mouse cells treated with BMP-2 or OP-1 (BMP-  
5 7) differentiate into astrocyte-like (glial) cells, and peripheral nerve regeneration using BMP-2 has been recently reported (Wang et al., WO 95/05846). In addition, BMP-4, BMP-5 and OP-1 (BMP-7) are expressed in epidermal ectoderm flanking the neural plate. Ectopic recombinant BMP-4 and OP-1 (BMP-7) proteins are capable of inducing neural plate cells to initiate dorsal neural cell fate differentiation (Liem et  
10 al., Cell, 82: 969-79 (1995)). At the spinal cord level, OP-1 and other BMPs, which should include the BMPs of the present invention, can induce neural crest cell differentiation. It is suggested that OP-1 and these BMPs can induce many or all dorsal neural cell types, including roof plate cells, neural crest cells, and commissural neurons, depending on localized positional cues. Therefore, additionally,  
15 morphogenic devices of this invention may also be implanted in or surrounding a joint for use in cartilage and soft tissue repair, or in or surrounding nervous system-associated tissue for use in neural regeneration and repair.

The tissue specificity of the particular morphogenic protein--or combination of morphogenic proteins with other biological factors--will determine the cell types or  
20 tissues that will be amenable to such treatments and can be selected by one skilled in the art. The ability to enhance other morphogenic protein-induced tissue regeneration by co-administering a BMP according to the present invention is thus not believed to be limited to any particular cell-type or tissue. It is envisioned that the invention as disclosed herein can be practiced to enhance the activities of new morphogenic  
25 proteins and to enhance new tissue inductive functions as they are discovered in the future.

The BMP compositions and devices comprising BMP will permit the physician to obtain predictable bone and/or cartilage formation. The BMP compositions and devices of this invention may be used to treat more efficiently  
30 and/or effectively all of the injuries, anomalies and disorders that have been described in the prior art of osteogenic devices. These include, for example, forming local bone

in fractures, non-union fractures, fusions and bony voids such as those created in tumor resections or those resulting from cysts; treating acquired and congenital craniofacial and other skeletal or dental anomalies (see e.g., Glowacki et al., *Lancet*, 1:959-63 (1981)); performing dental and periodontal reconstructions where lost bone replacement or bone augmentation is required such as in a jaw bone; and supplementing alveolar bone loss resulting from periodontal disease to delay or prevent tooth loss (see e.g., Sigurdsson et al., *J. Periodontol.*, 66:511-21 (1995)).

An osteogenic device of this invention which comprises a matrix comprising allogenic bone and a BMP may also be implanted at a site in need of bone replacement to accelerate allograft repair and incorporation in a mammal.

Another potential clinical application of the improved osteogenic devices of this invention is in cartilage repair, for example, following joint injury or in the treatment of osteoarthritis. The ability to enhance the cartilage-inducing activity of other morphogenic proteins by co-administering a BMP may permit faster or more extensive tissue repair and replacement using the same or lower levels of morphogenic proteins.

The BMP compositions and devices of this invention will be useful in treating certain congenital diseases and developmental abnormalities of cartilage, bone and other tissues. For example, homozygous OP-1 (BMP-7)-deficient mice die within 24 hours after birth due to kidney failure (Luo et al., *J. Bone Min. Res.*, 10 (Supp. 1):S163 (1995)). Kidney failure in these mice is associated with the failure to form renal glomeruli due to lack of mesenchymal tissue condensation. OP-1-deficient mice also have various skeletal abnormalities associated with their hindlimbs, rib cage and skull, are polydactyl, and exhibit aberrant retinal development. These results, in combination with those discussed above concerning the ability of OP-1 to induce differentiation into dorsal neural cell fates, indicate that OP-1 plays an important role in epithelial-mesenchymal interactions during development. It is anticipated that the compositions, devices and methods of this invention may be useful in the future for ameliorating these and other developmental abnormalities.

Developmental abnormalities of the bone may affect isolated or multiple regions of the skeleton or of a particular supportive or connective tissue type. These

abnormalities often require complicated bone transplantation procedures and orthopedic devices. The tissue repair and regeneration required after such procedures may occur more quickly and completely with the use of the BMPs of the present invention or the use of other morphogenic proteins used in combination with the

5 BMPs of the present invention.

Examples of heritable conditions, including congenital bone diseases, for which use of the morphogenic compositions and devices of this invention will be useful include osteogenesis imperfecta, the Hurler and Marfan syndromes, and several disorders of epiphyseal and metaphyseal growth centers such as is presented in

10 hypophosphatasia, a deficiency in alkaline phosphatase enzymatic activity.

Inflammatory joint diseases may also benefit from the improved BMP compositions and devices of this invention. These include but are not limited to infectious, non-infectious, rheumatoid and psoriatic arthritis, bursitis, ulcerative colitis, regional enteritis, Whipple's disease, and ankylosing spondylitis (also called

15 Marie Strumpell or Bechterew's disease); the so-called "collagen diseases" such as systemic lupus erythematosus (SLE), progressive systemic sclerosis (scleroderma), polymyositis (dermatomyositis), necrotizing vasculitides, Sjogren's syndrome (sicca syndrome), rheumatic fever, amyloidosis, thrombotic thrombocytopenic purpura and relapsing polychondritis. Heritable disorders of connective tissue include Marfan's

20 syndrome, homocystinuria, Ehlers-Danlos syndrome, osteogenesis imperfecta, alkaptonuria, pseudoxanthoma elasticum, cutis laxa, Hurler's syndrome, and myositis ossificans progressiva. The dosage regimen will be determined by the attending physician considering various factors which modify the action of the composition, e.g., amount of cartilaginous tissue desired to be formed the site of cartilaginous

25 tissue damage, the condition of the damaged cartilaginous tissue, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of additional proteins in the composition. The addition of other known growth factors, such as those discussed

30 *supra*, to the final composition, may also affect the dosage.



Progress can be monitored by periodic assessment of chondrocyte survival, cartilaginous tissue formation, or cartilaginous tissue growth and/or repair. The progress can be monitored by methods known in the art, for example, X-rays, arthroscopy, histomorphometric determinations and tetracycline labeling.

5

#### **Gene Therapy Methods**

Another aspect of the present invention is to gene therapy methods for treating disorders, diseases and conditions. Particularly preferred is a method for promoting the growth of endothelial cells, and more particularly vascular endothelial cells, and  
10 still more particularly for the stimulation of angiogenesis, using the BMP of the present invention in gene therapy. This method may be employed in treatment for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions. It may also be employed to stimulate angiogenesis and limb regeneration.

15 This method may also be employed for treating wounds due to injuries, burns, post-operative tissue repair, and ulcers since it has the ability to be mitogenic to various cells of different origins, such as fibroblast cells and skeletal muscle cells, and therefore, facilitate the repair or replacement of damaged or diseased tissue.

This method may also be employed to stimulate neuronal growth and to treat  
20 and prevent neuronal damage which occurs in certain neuronal disorders or neuro-degenerative conditions such as Alzheimer's disease, Parkinson's disease, and AIDS-related complex. Further, this method may have the ability to stimulate chondrocyte growth, therefore, may be employed to enhance bone and periodontal regeneration and aid in tissue transplants or bone grafts.

25 The gene therapy methods relate to the introduction of nucleic acid (DNA, RNA and antisense DNA or RNA) sequences into an animal to achieve expression of the BMP polypeptide of the present invention. This method requires a polynucleotide which codes for a BMP polypeptide operatively linked to a promoter and any other genetic elements necessary for the expression of the polypeptide by the target tissue.  
30 Such gene therapy and delivery techniques are known in the art, see, for example, WO90/11092, which is herein incorporated by reference.

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) comprising a promoter operably linked to a BMP polynucleotide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, see Beldegrun et al., *J. Natl. Cancer Inst.*, 85:207-216 (1993); Ferrantini et al., *Cancer Research*, 53:107-1112 (1993); Ferrantini et al., *J. Immunology* 153: 4604-4615 (1994); Kaido, T., et al., *Int. J. Cancer* 60: 221-229 (1995); Ogura, H., et al., *Cancer Research* 50: 5102-5106 (1990); Santodonato, L., et al., *Human Gene Therapy* 7:1-10 (1996); Santodonato, L., et al., *Gene Therapy* 4:1246-1255 (1997); and Zhang, J.-F. et al., *Cancer Gene Therapy* 3: 31-38 (1996)), which are herein incorporated by reference. In one embodiment, the cells which are engineered are arterial cells. The arterial cells may be reintroduced into the patient through direct injection to the artery, the tissues surrounding the artery, or through catheter injection.

As discussed in more detail below, the BMP polynucleotide constructs can be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, and the like). The BMP polynucleotide constructs may be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

In one embodiment, the BMP polynucleotide is delivered as a naked polynucleotide. The term "naked" polynucleotide, DNA or RNA refers to sequences that are free from any delivery vehicle that acts to assist, promote or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the BMP polynucleotides can also be delivered in liposome formulations and lipofectin formulations and the like can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Patent Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

The BMP polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Appropriate vectors include pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; pSVK3, pBPV, pMSG

and pSVL available from Pharmacia; and pEF1/V5, pcDNA3.1, and pRc/CMV2 available from Invitrogen. Other suitable vectors will be readily apparent to the skilled artisan.

Any strong promoter known to those skilled in the art can be used for driving the expression of BMP DNA. Suitable promoters include adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs; the b-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter for BMP.

Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The BMP polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated

cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked acid sequence injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 mg/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration.

The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked BMP DNA constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The naked polynucleotides are delivered by any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, and so-called "gene guns". These delivery methods are known in the art.

As is evidenced in the Examples, naked BMP nucleic acid sequences can be administered *in vivo* results in the successful expression of BMP polypeptide in the femoral arteries of rabbits.

The constructs may also be delivered with delivery vehicles such as viral sequences, viral particles, liposome formulations, lipofectin, precipitating agents, etc. Such methods of delivery are known in the art.

In certain embodiments, the BMP polynucleotide constructs are complexed in a liposome preparation. Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic liposomes are particularly preferred because a tight charge complex can be formed between the cationic liposome and the polyanionic

nucleic acid. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., *Proc. Natl. Acad. Sci. USA*, 84:7413-7416 (1987), which is herein incorporated by reference); mRNA (Malone et al., *Proc. Natl. Acad. Sci. USA*, 86:6077-6081 (1989), which is herein incorporated by reference); and  
5 purified transcription factors (Debs et al., *J. Biol. Chem.*, 265:10189-10192 (1990), which is herein incorporated by reference), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are particularly useful and are available under the trademark Lipofectin, from GIBCO  
10 BRL, Grand Island, N.Y. (See, also, Felgner et al., *Proc. Natl. Acad. Sci. USA*, 84:7413-7416 (1987), which is herein incorporated by reference). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer).

Other cationic liposomes can be prepared from readily available materials  
15 using techniques well known in the art. See, e.g. PCT Publication NO: WO 90/11092 (which is herein incorporated by reference) for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Preparation of DOTMA liposomes is explained in the literature, see, e.g., P. Felgner et al., *Proc. Natl. Acad. Sci. USA*, 84:7413-7417, which is herein incorporated by reference.

20 Similar methods can be used to prepare liposomes from other cationic lipid materials.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, Ala.), or can be easily prepared using readily available materials. Such materials include phosphatidyl, choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC),  
25 dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

For example, commercially dioleoylphosphatidyl choline (DOPC),  
30 dioleoylphosphatidyl glycerol (DOPG), and dioleoylphosphatidyl ethanolamine (DOPE) can be used in various combinations to make conventional liposomes, with

or without the addition of cholesterol. Thus, for example, DOPG/DOPC vesicles can be prepared by drying 50 mg each of DOPG and DOPC under a stream of nitrogen gas into a sonication vial. The sample is placed under a vacuum pump overnight and is hydrated the following day with deionized water. The sample is then sonicated for  
5 2 hours in a capped vial, using a Heat Systems model 350 sonicator equipped with an inverted cup (bath type) probe at the maximum setting while the bath is circulated at 15EC. Alternatively, negatively charged vesicles can be prepared without sonication to produce multilamellar vesicles or by extrusion through nucleopore membranes to produce unilamellar vesicles of discrete size. Other methods are known and available  
10 to those of skill in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs), with SUVs being preferred. The various liposome-nucleic acid complexes are prepared using methods well known in the art. See, e.g., Straubinger et al., *Methods of Immunology*, 101:512-527 (1983),  
15 which is herein incorporated by reference. For example, MLVs containing nucleic acid can be prepared by depositing a thin film of phospholipid on the walls of a glass tube and subsequently hydrating with a solution of the material to be encapsulated. SUVs are prepared by extended sonication of MLVs to produce a homogeneous population of unilamellar liposomes. The material to be entrapped is added to a  
20 suspension of preformed MLVs and then sonicated. When using liposomes containing cationic lipids, the dried lipid film is resuspended in an appropriate solution such as sterile water or an isotonic buffer solution such as 10 mM Tris/NaCl, sonicated, and then the preformed liposomes are mixed directly with the DNA. The liposome and DNA form a very stable complex due to binding of the positively charged liposomes  
25 to the cationic DNA. SUVs find use with small nucleic acid fragments. LUVs are prepared by a number of methods, well known in the art. Commonly used methods include  $\text{Ca}^{2+}$ -EDTA chelation (Papahadjopoulos et al., *Biochim. Biophys. Acta*, 394:483 (1975); Wilson et al., *Cell*, 17:77 (1979)); ether injection (Deamer et al., *Biochim. Biophys. Acta*, 443:629 (1976); Ostro et al., *Biochem. Biophys. Res.*  
30 *Commun.*, 76:836 (1977); Fraley et al., *Proc. Natl. Acad. Sci. USA*, 76:3348 (1979)); detergent dialysis (Enoch et al., *Proc. Natl. Acad. Sci. USA*, 76:145 (1979)); and

reverse-phase evaporation (REV) (Fraley et al., *J. Biol. Chem.*, 255:10431 (1980); Szoka et al., *Proc. Natl. Acad. Sci. USA*, 75:145 (1978); Schaefer-Ridder et al., *Science*, 215:166 (1982)), which are herein incorporated by reference.

Generally, the ratio of DNA to liposomes will be from about 10:1 to about 1:10. Preferably, the ration will be from about 5:1 to about 1:5. More preferably, the ration will be about 3:1 to about 1:3. Still more preferably, the ratio will be about 1:1.

U.S. Patent NO: 5,676,954 (which is herein incorporated by reference) reports on the injection of genetic material, complexed with cationic liposomes carriers, into mice. U.S. Patent Nos. 4,897,355, 4,946,787, 5,049,386, 5,459,127, 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication NO: WO 94/9469 (which are herein incorporated by reference) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication NO: WO 94/9469 (which are herein incorporated by reference) provide methods for delivering DNA-cationic lipid complexes to mammals.

In certain embodiments, cells are be engineered, *ex vivo* or *in vivo*, using a retroviral particle containing RNA which comprises a sequence encoding BMP. Retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, Rous sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, R-2, R-AM, PA12, T19-14X, VT-19-17-H2, RCRE, RCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy*, 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO<sub>4</sub> precipitation. In one alternative, the retroviral

plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include polynucleotide encoding BMP. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express BMP.

In certain other embodiments, cells are engineered, *ex vivo* or *in vivo*, with BMP polynucleotide contained in an adenovirus vector. Adenovirus can be manipulated such that it encodes and expresses BMP, and at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. Adenovirus expression is achieved without integration of the viral DNA into the host cell chromosome, thereby alleviating concerns about insertional mutagenesis. Furthermore, adenoviruses have been used as live enteric vaccines for many years with an excellent safety profile (Schwartz et al., *Am. Rev. Respir. Dis.*, 109:233-238 (1974)). Finally, adenovirus mediated gene transfer has been demonstrated in a number of instances including transfer of alpha-1-antitrypsin and CFTR to the lungs of cotton rats (Rosenfeld et al., *Science*, 252:431-434 (1991); Rosenfeld et al., *Cell*, 68:143-155 (1992)). Furthermore, extensive studies to attempt to establish adenovirus as a causative agent in human cancer were uniformly negative (Green et al. *Proc. Natl. Acad. Sci. USA*, 76:6606 (1979)).

Suitable adenoviral vectors useful in the present invention are described, for example, in Kozarsky and Wilson, *Curr. Opin. Genet. Devel.*, 3:499-503 (1993); Rosenfeld et al., *Cell*, 68:143-155 (1992); Engelhardt et al., *Human Genet. Ther.*, 4:759-769 (1993); Yang et al., *Nature Genet.*, 7:362-369 (1994); Wilson et al., *Nature*, 365:691-692 (1993); and U.S. Patent NO: 5,652,224, which are herein incorporated by reference. For example, the adenovirus vector Ad2 is useful and can be grown in human 293 cells. These cells contain the E1 region of adenovirus and constitutively express Ela and Elb, which complement the defective adenoviruses by providing the products of the genes deleted from the vector. In addition to Ad2, other varieties of adenovirus (e.g., Ad3, Ad5, and Ad7) are also useful in the present invention.



Preferably, the adenoviruses used in the present invention are replication deficient. Replication deficient adenoviruses require the aid of a helper virus and/or packaging cell line to form infectious particles. The resulting virus is capable of infecting cells and can express a polynucleotide of interest which is operably linked to a promoter, for example, the HARP promoter of the present invention, but cannot replicate in most cells. Replication deficient adenoviruses may be deleted in one or more of all or a portion of the following genes: E1a, E1b, E3, E4, E2a, or L1 through L5.

In certain other embodiments, the cells are engineered, *ex vivo* or *in vivo*, using an adeno-associated virus (AAV). AAVs are naturally occurring defective viruses that require helper viruses to produce infectious particles (Muzyczka, *Curr. Topics in Microbiol. Immunol.*, 158:97 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5 kb. Methods for producing and using such AAVs are known in the art. See, for example, U.S. Patent Nos. 5,139,941, 5,173,414, 5,354,678, 5,436,146, 5,474,935, 5,478,745, and 5,589,377.

For example, an appropriate AAV vector for use in the present invention will include all the sequences necessary for DNA replication, encapsidation, and host-cell integration. The BMP polynucleotide construct is inserted into the AAV vector using standard cloning methods, such as those found in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (1989). The recombinant AAV vector is then transfected into packaging cells which are infected with a helper virus, using any standard technique, including lipofection, electroporation, calcium phosphate precipitation, etc. Appropriate helper viruses include adenoviruses, cytomegaloviruses, vaccinia viruses, or herpes viruses. Once the packaging cells are transfected and infected, they will produce infectious AAV viral particles which contain the BMP polynucleotide construct. These viral particles are then used to transduce eukaryotic cells, either *ex vivo* or *in vivo*. The transduced cells will contain the BMP polynucleotide construct integrated into its genome, and will express BMP.

Another method of gene therapy involves operably associating heterologous control regions and endogenous polynucleotide sequences (e.g. encoding BMP) via homologous recombination (see, e.g., U.S. Patent NO: 5,641,670, issued June 24, 1997; International Publication NO: WO 96/29411, published September 26, 1996; 5 International Publication NO: WO 94/12650, published August 4, 1994; Koller et al., *Proc. Natl. Acad. Sci. USA*, 86:8932-8935 (1989); and Zijlstra et al., *Nature*, 342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not normally expressed in the cells, or is expressed at a lower level than desired.

10 Polynucleotide constructs are made, using standard techniques known in the art, which contain the promoter with targeting sequences flanking the promoter. Suitable promoters are described herein. The targeting sequence is sufficiently complementary to an endogenous sequence to permit homologous recombination of the promoter-targeting sequence with the endogenous sequence. The targeting 15 sequence will be sufficiently near the 5' end of the BMP desired endogenous polynucleotide sequence so the promoter will be operably linked to the endogenous sequence upon homologous recombination.

The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' 20 and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter. The amplified promoter and targeting sequences are digested and ligated together.

25 The promoter-targeting sequence construct is delivered to the cells, either as naked polynucleotide, or in conjunction with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, whole viruses, lipofection, precipitating agents, etc., described in more detail above. The P promoter-targeting sequence can be delivered by any method, included direct needle injection, intravenous injection, 30 topical administration, catheter infusion, particle accelerators, etc. The methods are described in more detail below.

The promoter-targeting sequence construct is taken up by cells. Homologous recombination between the construct and the endogenous sequence takes place, such that an endogenous BMP sequence is placed under the control of the promoter. The promoter then drives the expression of the endogenous BMP sequence.

5       The polynucleotides encoding polypeptides of the present invention may be administered along with other polynucleotides encoding other angiogenic proteins. Angiogenic proteins include, but are not limited to, acidic and basic fibroblast growth factors, VEGF-1, epidermal growth factor alpha and beta, platelet-derived endothelial cell growth factor, platelet-derived growth factor, tumor necrosis factor alpha,  
10   hepatocyte growth factor, insulin like growth factor, colony stimulating factor, macrophage colony stimulating factor, granulocyte/macrophage colony stimulating factor, and nitric oxide synthase.

Preferably, the polynucleotide encoding an BMP contains a secretory signal sequence that facilitates secretion of the protein. Typically, the signal sequence is  
15   positioned in the coding region of the polynucleotide to be expressed towards or at the 5' end of the coding region. The signal sequence may be homologous or heterologous to the polynucleotide of interest and may be homologous or heterologous to the cells to be transfected. Additionally, the signal sequence may be chemically synthesized using methods known in the art.

20       Any mode of administration of any of the above-described polynucleotides constructs can be used so long as the mode results in the expression of one or more molecules in an amount sufficient to provide a therapeutic effect. This includes direct needle injection, systemic injection, catheter infusion, biolistic injectors, particle accelerators (i.e., "gene guns"), gelfoam sponge depots, other commercially available  
25   depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. For example, direct injection of naked calcium phosphate-precipitated plasmid into rat liver and rat spleen or a protein-coated plasmid into the portal vein has resulted in gene expression of the foreign gene in the  
30   rat livers. (Kaneda et al., *Science*, 243:375 (1989)).

A preferred method of local administration is by direct injection. Preferably, a recombinant molecule of the present invention complexed with a delivery vehicle is administered by direct injection into or locally within the area of arteries.

Administration of a composition locally within the area of arteries refers to injecting  
5 the composition centimeters and preferably, millimeters within arteries.

Another method of local administration is to contact a polynucleotide construct of the present invention in or around a surgical wound. For example, a patient can undergo surgery and the polynucleotide construct can be coated on the surface of tissue inside the wound or the construct can be injected into areas of tissue  
10 inside the wound.

Therapeutic compositions useful in systemic administration, include recombinant molecules of the present invention complexed to a targeted delivery vehicle of the present invention. Suitable delivery vehicles for use with systemic administration comprise liposomes comprising ligands for targeting the vehicle to a  
15 particular site.

Preferred methods of systemic administration, include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be performed using methods standard in the art. Aerosol delivery can also be performed using methods standard in the art (see, for example, Stribling et al., *Proc. Natl. Acad. Sci. USA*, 189:11277-11281 (1992), which is incorporated herein by reference). Oral  
20 delivery can be performed by complexing a polynucleotide construct of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical delivery can be performed by mixing a  
25 polynucleotide construct of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the animal, the precise  
30 condition requiring treatment and its severity, and the route of administration. The frequency of treatments depends upon a number of factors, such as the amount of

polynucleotide constructs administered per dose, as well as the health and history of the subject. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian. Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals and  
5 birds. Preferred mammals include humans, dogs, cats, mice, rats, rabbits sheep, cattle, horses and pigs, with humans being particularly

### **Therapeutic Uses**

The BMP proteins of the present invention may be used as mitogens for  
10 vascular and lymphatic endothelial cells. Accordingly, BMP polypeptides, or biologically active portions thereof, may be employed to treat vascular trauma by promoting angiogenesis. For example, to stimulate the growth of transplanted tissue where coronary bypass surgery is performed. BMP polypeptides, or biologically active portions thereof, may also be employed to promote wound healing, particularly  
15 to re-vascularize damaged tissues or stimulate collateral blood flow during ischemia and where new capillary angiogenesis is desired. BMP polypeptides, or biologically active portions thereof, may be employed to treat full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, BMP polypeptides, or biologically active portions thereof, may be employed  
20 to treat full-thickness burns and injuries where a skin graft or flap is used to repair such burns and injuries. BMP polypeptides, or biologically active portions thereof, may also be employed for use in plastic surgery, for example, for the repair of lacerations, burns, or other trauma. In addition, BMP polypeptides, or biologically active portions thereof, can be used to promote healing of wounds and injuries to the  
25 eye as well as to treat eye diseases.

Along these same lines, BMP polypeptides, or biologically active portions thereof, may also be employed to induce the growth of damaged bone, periodontium or ligament tissue. BMP polypeptides, or biologically active portions thereof, may also be employed for regenerating supporting tissues of the teeth, including cementum  
30 and periodontal ligament, that have been damaged by, *e.g.*, periodontal disease or trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, BMP polypeptides, or biologically active portions thereof, may be employed in association with surgery and following the repair of incisions and cuts. BMP polypeptides, or biologically active portions thereof, may also be employed for the treatment of abdominal wounds where there is a high risk of infection.

BMP polypeptides, or biologically active portions thereof, may be employed for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, BMP polypeptides, or biologically active portions thereof, can be applied to the surface of the graft or at the junction to promote the growth of vascular endothelial cells. BMP polypeptides, or biologically active portions thereof, may also be employed to repair damage of myocardial tissue as a result of myocardial infarction. BMP polypeptides, or biologically active portions thereof, may also be employed to repair the cardiac vascular system after ischemia. BMP polypeptides, or biologically active portions thereof, may also be employed to treat damaged vascular tissue as a result of coronary artery disease and peripheral and CNS vascular disease.

BMP polypeptides, or biologically active portions thereof, may also be employed to coat artificial prostheses or natural organs which are to be transplanted in the body to minimize rejection of the transplanted material and to stimulate vascularization of the transplanted materials.

BMP polypeptides, or biologically active portions thereof, may also be employed for vascular tissue repair of injuries resulting from trauma, for example, that occurring during arteriosclerosis and required following balloon angioplasty where vascular tissues are damaged.

BMP polypeptides, or biologically active portions thereof, may also be used to treat peripheral arterial disease. Accordingly, in a further aspect, there is provided a process for utilizing BMP polypeptides, or biologically active portions thereof, to treat peripheral arterial disease. Preferably, a BMP polypeptide is administered to an individual for the purpose of alleviating or treating peripheral arterial disease.

Suitable doses, formulations, and administration routes are described below.

BMP polypeptides, or biologically active portions thereof, may also be used to promote the endothelial function of lymphatic tissues and vessels, such as to treat the loss of lymphatic vessels, occlusions of lymphatic vessels, and lymphangiomas. BMP polypeptides may also be used to stimulate lymphocyte production.

5 BMP polypeptides, or biologically active portions thereof, may also be used to treat hemangioma in newborns. Accordingly, in a further aspect, there is provided a process for utilizing BMP polypeptides to treat hemangioma in newborns. Preferably, a BMP polypeptide is administered to an individual for the purpose of alleviating or treating hemangioma in newborns. Suitable doses, formulations, and administration  
10 routes are described below.

BMP polypeptides, or biologically active portions thereof, may also be used to prevent or treat abnormal retinal development in premature newborns. Accordingly, in a further aspect, there is provided a process for utilizing BMP polypeptides to treat abnormal retinal development in premature newborns. Preferably, a BMP  
15 polypeptide is administered to an individual for the purpose of alleviating or treating abnormal retinal development in premature newborns. Suitable doses, formulations, and administration routes are described below.

BMP polypeptides, or biologically active portions thereof, may be used to treat primary (idiopathic) lymphedemas, including Milroy's disease and Lymphedema  
20 praecox. Accordingly, in a further aspect, there is provided a process for utilizing BMP polypeptides to treat primary (idiopathic) lymphedemas, including Milroy's disease and Lymphedema praecox. Preferably, an BMP polypeptide is administered to an individual for the purpose of alleviating or treating primary (idiopathic) lymphedemas, including Milroy's disease and Lymphedema praecox. BMP  
25 polypeptides may also be used to treat edema as well as to effect blood pressure in an animal. Suitable doses, formulations, and administration routes are described below.

BMP polypeptides, or biologically active portions thereof, may also be used to treat secondary (obstructive) lifetimes including those that result from (i) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer,  
30 and (iii) trauma and infection. Accordingly, in a further aspect, there is provided a process for utilizing BMP polypeptides to treat secondary (obstructive) lifetimes

including those that result from (I) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Preferably, a BMP polypeptide is administered to an individual for the purpose of secondary (obstructive) lifetimes including those that result from (I) the removal of lymph nodes and vessels, (ii) radiotherapy and surgery in the treatment of cancer, and (iii) trauma and infection. Suitable doses, formulations, and administration routes are described below.

BMP polypeptides, or biologically active portions thereof, may also be used to treat Kaposi's Sarcoma. Accordingly, in a further aspect, there is provided a process for utilizing BMP polypeptides to treat Kaposi's Sarcoma. Preferably, a BMP polypeptide is administered to an individual for the purpose of alleviating or treating Kaposi's Sarcoma. Suitable doses, formulations, and administration routes are described below.

#### **Biological Activities**

The polynucleotides and polypeptides of the present invention can be used in assays to test for one or more biological activities of BMP activity. If these polynucleotides and polypeptides do exhibit activity in a particular assay, it is likely that these molecules may be involved in the diseases associated with the biological activity. Thus, the polynucleotides and polypeptides could be used to treat the associated disease.

#### **Immune Activity**

A polypeptide or polynucleotide of the present invention may be useful in treating deficiencies or disorders of the immune system, by activating or inhibiting the proliferation, differentiation, or mobilization (chemotaxis) of immune cells. Immune cells develop through a process called hematopoiesis, producing myeloid (platelets, red blood cells, neutrophils, and macrophages) and lymphoid (B and T lymphocytes) cells from pluripotent stem cells. The etiology of these immune deficiencies or disorders may be genetic, somatic, such as cancer or some autoimmune disorders, acquired (e.g., by chemotherapy or toxins), or infectious.



Moreover, a polynucleotide or polypeptide of the present invention can be used as a marker or detector of a particular immune system disease or disorder.

A polynucleotide or polypeptide of the present invention may be useful in treating or detecting deficiencies or disorders of hematopoietic cells. A

5 polypeptide or polynucleotide of the present invention could be used to increase differentiation and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat those disorders associated with a decrease in certain (or many) types hematopoietic cells. Examples of immunologic deficiency syndromes include, but are not limited to: blood protein disorders (e.g. agammaglobulinemia, dysgammaglobulinemia), ataxia telangiectasia, common variable immunodeficiency, 10 Digeorge Syndrome, HIV infection, HTLV-BLV infection, leukocyte adhesion deficiency syndrome, lymphopenia, phagocyte bactericidal dysfunction, severe combined immunodeficiency (SCIDs), Wiskott-Aldrich Disorder, anemia, thrombocytopenia, or hemoglobinuria.

15 Moreover, a polypeptide or polynucleotide of the present invention could also be used to modulate hemostatic (the stopping of bleeding) or thrombolytic activity (clot formation). For example, by increasing hemostatic or thrombolytic activity, a polynucleotide or polypeptide of the present invention could be used to treat blood coagulation disorders (e.g., afibrinogenemia, factor deficiencies), blood platelet 20 disorders (e.g. thrombocytopenia), or wounds resulting from trauma, surgery, or other causes. Alternatively, a polynucleotide or polypeptide of the present invention that can decrease hemostatic or thrombolytic activity could be used to inhibit or dissolve clotting. These molecules could be important in the treatment of heart attacks (infarction), strokes, or scarring.

25 A polynucleotide or polypeptide of the present invention may also be useful in treating or detecting autoimmune disorders. Many autoimmune disorders result from inappropriate recognition of self as foreign material by immune cells. This inappropriate recognition results in an immune response leading to the destruction of the host tissue. Therefore, the administration of a polypeptide or polynucleotide of the 30 present invention that inhibits an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing

autoimmune disorders. For example, soluble forms of the polynucleotides of the present invention may be useful in inhibiting cytokine activity by absorption.

Examples of autoimmune disorders that can be treated or detected by the present invention include, but are not limited to: Addison's Disease, hemolytic  
5 anemia, antiphospholipid syndrome, rheumatoid arthritis, dermatitis, allergic encephalomyelitis, glomerulonephritis, Goodpasture's Syndrome, Graves' Disease, Multiple Sclerosis, Myasthenia Gravis, Neuritis, Ophthalmia, Bullous Pemphigoid, Pemphigus, Polyendocrinopathies, Purpura, Reiter's Disease, Stiff-Man Syndrome, Autoimmune Thyroiditis, Systemic Lupus Erythematosus, Autoimmune Pulmonary  
10 Inflammation, Guillain-Barre Syndrome, insulin dependent diabetes mellitis, and autoimmune inflammatory eye disease.

Similarly, allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems, may also be treated by a polypeptide or polynucleotide of the present invention. Moreover, these molecules can be used to  
15 treat anaphylaxis, hypersensitivity to an antigenic molecule, or blood group incompatibility.

A polynucleotide or polypeptide of the present invention may also be used to treat and/or prevent organ rejection or graft-versus-host disease (GVHD). Organ rejection occurs by host immune cell destruction of the transplanted tissue through an  
20 immune response. Similarly, an immune response is also involved in GVHD, but, in this case, the foreign transplanted immune cells destroy the host tissues. The administration of a polypeptide or polynucleotide of the present invention that inhibits an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing organ rejection or GVHD.

25 Similarly, a polypeptide or polynucleotide of the present invention may also be used to modulate inflammation. For example, the polypeptide or polynucleotide may inhibit the proliferation and differentiation of cells involved in an inflammatory response. These molecules can be used to treat inflammatory conditions, both chronic and acute conditions, including inflammation associated with infection (e.g., septic  
30 shock, sepsis, or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute

rejection, nephritis, cytokine or chemokine induced lung injury, inflammatory bowel disease, Crohn's disease, or resulting from over production of cytokines (e.g., TNF or IL-1.)

## 5 **Cardiovascular Disorders**

BMP polynucleotides or polypeptides may be used to treat cardiovascular disorders, including peripheral artery disease, such as limb ischemia.

Cardiovascular disorders include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital  
10 heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus,  
15 and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogly of Fallot, ventricular heart septal defects.

Cardiovascular disorders also include heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart  
20 failure, congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium,  
25 postpericardiotomy syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial  
30 block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus

syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, 5 Torsades de Pointes, and ventricular tachycardia.

Heart valve disease include aortic valve insufficiency, aortic valve stenosis, hear murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve 10 insufficiency, and tricuspid valve stenosis.

Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial 15 reperfusion injury, and myocarditis.

Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

Cardiovascular diseases also include vascular diseases such as aneurysms, 20 angiodyplasia, angiomatosis, bacillary angiomatosis, Hippel-Lindau Disease, Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, 25 hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis, pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, atacia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous 30 insufficiency.

Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

Arterial occlusive diseases include arteriosclerosis, intermittent claudication,  
5 carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

Cerebrovascular disorders include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral  
10 arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subarachnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache,  
15 migraine, and vertebrobasilar insufficiency.

Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromboembolisms. Thrombosis include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis,  
20 Wallenberg's syndrome, and thrombophlebitis.

Ischemia includes cerebral ischemia, critical limb ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node  
25 syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

BMP polypeptides may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle  
30 accelerators, gelfoam sponge depots, other commercially available depot materials, osmotic pumps, oral or suppository solid pharmaceutical formulations, decanting or

topical applications during surgery, aerosol delivery. Such methods are known in the art. BMP polypeptides may be administered as part of a pharmaceutical composition, described in more detail below. Methods of delivering BMP polynucleotides are described in more detail herein.

5

#### **Wound Healing and Epithelial Cell Proliferation**

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing BMP polynucleotides or polypeptides of the present invention for therapeutic purposes, for example, to stimulate epithelial cell proliferation and basal keratinocytes for the purpose of wound healing, and to stimulate hair follicle production and healing of dermal wounds. BMP polynucleotides or polypeptides may be clinically useful in stimulating wound healing including surgical wounds, excisional wounds, deep wounds involving damage of the dermis and epidermis, eye tissue wounds, dental tissue wounds, oral cavity wounds, diabetic ulcers, dermal ulcers, cubitus ulcers, arterial ulcers, venous stasis ulcers, burns resulting from heat exposure or chemicals, and other abnormal wound healing conditions such as uremia, malnutrition, vitamin deficiencies and complications associated with systemic treatment with steroids, radiation therapy and antineoplastic drugs and antimetabolites. BMP polynucleotides or polypeptides could be used to promote dermal reestablishment subsequent to dermal loss.

BMP polynucleotides or polypeptides could be used to increase the adherence of skin grafts to a wound bed and to stimulate re-epithelialization from the wound bed. The following are types of grafts that BMP polynucleotides or polypeptides could be used to increase adherence to a wound bed: autografts, artificial skin, allografts, autodermic graft, autoepdermic grafts, avacular grafts, Blair-Brown grafts, bone graft, brephoplastic grafts, cutis graft, delayed graft, dermic graft, epidermic graft, fascia graft, full thickness graft, heterologous graft, xenograft, homologous graft, hyperplastic graft, lamellar graft, mesh graft, mucosal graft, Ollier-Thiersch graft, omenpal graft, patch graft, pedicle graft, penetrating graft, split skin graft, thick split graft. BMP polynucleotides or polypeptides can be used to promote skin strength and to improve the appearance of aged skin.

30

It is thought that BMP polynucleotides or polypeptides will also produce changes in hepatocyte proliferation, and epithelial cell proliferation in the lung, breast, pancreas, stomach, small intestine, and large intestine. BMP polynucleotides or polypeptides could promote proliferation of epithelial cells such as sebocytes, hair  
5 follicles, hepatocytes, type II pneumocytes, mucin-producing goblet cells, and other epithelial cells and their progenitors contained within the skin, lung, liver, and gastrointestinal tract. BMP polynucleotides or polypeptides may promote proliferation of endothelial cells, keratinocytes, and basal keratinocytes.

BMP polynucleotides or polypeptides could also be used to reduce the side  
10 effects of gut toxicity that result from radiation, chemotherapy treatments or viral infections. BMP polynucleotides or polypeptides may have a cytoprotective effect on the small intestine mucosa. BMP polynucleotides or polypeptides may also stimulate healing of mucositis (mouth ulcers) that result from chemotherapy and viral infections.

BMP polynucleotides or polypeptides could further be used in full  
15 regeneration of skin in full and partial thickness skin defects, including burns, (i.e., repopulation of hair follicles, sweat glands, and sebaceous glands), treatment of other skin defects such as psoriasis. BMP polynucleotides or polypeptides could be used to treat epidermolysis bullosa, a defect in adherence of the epidermis to the underlying  
20 dermis which results in frequent, open and painful blisters by accelerating reepithelialization of these lesions. BMP polynucleotides or polypeptides could also be used to treat gastric and duodenal ulcers and help heal by scar formation of the mucosal lining and regeneration of glandular mucosa and duodenal mucosal lining more rapidly. Inflammatory bowel diseases, such as Crohn's disease and ulcerative  
25 colitis, are diseases which result in destruction of the mucosal surface of the small or large intestine, respectively. Thus, BMP polynucleotides or polypeptides could be used to promote the resurfacing of the mucosal surface to aid more rapid healing and to prevent progression of inflammatory bowel disease. Treatment with BMP  
polynucleotides or polypeptides is expected to have a significant effect on the  
30 production of mucus throughout the gastrointestinal tract and could be used to protect the intestinal mucosa from injurious substances that are ingested or following surgery.

BMP polynucleotides or polypeptides could be used to treat diseases associate with the under expression of angiogenic polypeptides.

Moreover, BMP polynucleotides or polypeptides could be used to prevent and heal damage to the lungs due to various pathological states. A growth factor such as

5 BMP polynucleotides or polypeptides which could stimulate proliferation and differentiation and promote the repair of alveoli and bronchiolar epithelium to prevent or treat acute or chronic lung damage. For example, emphysema, which results in the progressive loss of aveoli, and inhalation injuries, i.e., resulting from smoke inhalation and burns, that cause necrosis of the bronchiolar epithelium and alveoli

10 could be effectively treated using BMP polynucleotides or polypeptides. Also, BMP polynucleotides or polypeptides could be used to stimulate the proliferation of and differentiation of type II pneumocytes, which may help treat or prevent disease such as hyaline membrane diseases, such as infant respiratory distress syndrome and bronchopulmonary dysplasia, in premature infants.

15 BMP polynucleotides or polypeptides could stimulate the proliferation and differentiation of hepatocytes and, thus, could be used to alleviate or treat liver diseases and pathologies such as fulminant liver failure caused by cirrhosis, liver damage caused by viral hepatitis and toxic substances (i.e., acetaminophen, carbon tetrahaloride and other hepatotoxins known in the art).

20 In addition, BMP polynucleotides or polypeptides could be used treat or prevent the onset of diabetes mellitus. In patients with newly diagnosed Types I and II diabetes, where some islet cell function remains, BMP polynucleotides or polypeptides could be used to maintain the islet function so as to alleviate, delay or prevent permanent manifestation of the disease. Also, BMP polynucleotides or

25 polypeptides could be used as an auxiliary in islet cell transplantation to improve or promote islet cell function.

### **Hyperproliferative Disorders**

A polypeptide or polynucleotide can be used to treat or detect

30 hyperproliferative disorders, including neoplasms. A polypeptide or polynucleotide of the present invention may inhibit the proliferation of the disorder through direct or



indirect interactions. Alternatively, a polypeptide or polynucleotide of the present invention may proliferate other cells which can inhibit the hyperproliferative disorder.

For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, 5 differentiating, or mobilizing T-cells, hyperproliferative disorders can be treated. This immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, decreasing an immune response may also be a method of treating hyperproliferative disorders, such as a chemotherapeutic agent.

10 Examples of hyperproliferative disorders that can be treated or detected by a polynucleotide or polypeptide of the present invention include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous (central and peripheral), lymphatic 15 system, pelvic, skin, soft tissue, spleen, thoracic, and urogenital.

Similarly, other hyperproliferative disorders can also be treated or detected by a polynucleotide or polypeptide of the present invention. Examples of such hyperproliferative disorders include, but are not limited to: 20 hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenström's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

### Infectious Disease

25 A polypeptide or polynucleotide of the present invention can be used to treat or detect infectious agents. For example, by increasing the immune response, particularly increasing the proliferation and differentiation of B and/or T cells, infectious diseases may be treated. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. 30 Alternatively, the polypeptide or polynucleotide of the present invention may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

Viruses are one example of an infectious agent that can cause disease or symptoms that can be treated or detected by a polynucleotide or polypeptide of the present invention. Examples of viruses, include, but are not limited to the following DNA and RNA viral families: Arbovirus, Adenoviridae, Arenaviridae, Arterivirus, 5 Birnaviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Flaviviridae, Hepadnaviridae (Hepatitis), Herpesviridae (such as, Cytomegalovirus, Herpes Simplex, Herpes Zoster), Mononegavirus (e.g., Paramyxoviridae, Morbillivirus, Rhabdoviridae), Orthomyxoviridae (e.g., Influenza), Papovaviridae, Parvoviridae, Picornaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (e.g., 10 Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus), and Togaviridae (e.g., Rubivirus). Viruses falling within these families can cause a variety of diseases or symptoms, including, but not limited to: arthritis, bronchiolitis, encephalitis, eye infections (e.g., conjunctivitis, keratitis), chronic fatigue syndrome, hepatitis (A, B, C, E, Chronic Active, Delta), meningitis, opportunistic infections (e.g., AIDS), 15 pneumonia, Burkitt's Lymphoma, chickenpox, hemorrhagic fever, Measles, Mumps, Parainfluenza, Rabies, the common cold, Polio, leukemia, Rubella, sexually transmitted diseases, skin diseases (e.g., Kaposi's, warts), and viremia. A polypeptide or polynucleotide of the present invention can be used to treat or detect any of these symptoms or diseases.

20 Similarly, bacterial or fungal agents that can cause disease or symptoms and that can be treated or detected by a polynucleotide or polypeptide of the present invention include, but not limited to, the following Gram-Negative and Gram-positive bacterial families and fungi: Actinomycetales (e.g., Corynebacterium, Mycobacterium, Nocardia), Aspergillosis, Bacillaceae (e.g., Anthrax, Clostridium), 25 Bacteroidaceae, Blastomycosis, Bordetella, Borrelia, Brucellosis, Candidiasis, Campylobacter, Coccidioidomycosis, Cryptococcosis, Dermatocycoses, Enterobacteriaceae (Klebsiella, Salmonella, Serratia, Yersinia), Erysipelothrix, Helicobacter, Legionellosis, Leptospirosis, Listeria, Mycoplasmatales, Neisseriaceae (e.g., Acinetobacter, Gonorrhea, Meningococcal), Pasteurellaceae Infections (e.g., 30 Actinobacillus, Haemophilus, Pasteurella), Pseudomonas, Rickettsiaceae, Chlamydiaceae, Syphilis, and Staphylococcal. These bacterial or fungal families can

cause the following diseases or symptoms, including, but not limited to: bacteremia, endocarditis, eye infections (conjunctivitis, tuberculosis, uveitis), gingivitis, opportunistic infections (e.g., AIDS related infections), paronychia, prosthesis-related infections, Reiter's Disease, respiratory tract infections, such as Whooping Cough or  
5 Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, Dysentery, Paratyphoid Fever, food poisoning, Typhoid, pneumonia, Gonorrhea, meningitis, Chlamydia, Syphilis, Diphtheria, Leprosy, Paratuberculosis, Tuberculosis, Lupus, Botulism, gangrene, tetanus, impetigo, Rheumatic Fever, Scarlet Fever, sexually transmitted diseases, skin diseases (e.g., cellulitis, dermatocycoses), toxemia, urinary tract infections, wound  
10 infections. A polypeptide or polynucleotide of the present invention can be used to treat or detect any of these symptoms or diseases.

Moreover, parasitic agents causing disease or symptoms that can be treated or detected by a polynucleotide or polypeptide of the present invention include, but not limited to, the following families: Amebiasis, Babesiosis, Coccidiosis,  
15 Cryptosporidiosis, Dientamoebiasis, Dourine, Ectoparasitic, Giardiasis, Helminthiasis, Leishmaniasis, Theileriasis, Toxoplasmosis, Trypanosomiasis, and Trichomonas. These parasites can cause a variety of diseases or symptoms, including, but not limited to: Scabies, Trombiculiasis, eye infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic  
20 infections (e.g., AIDS related), Malaria, pregnancy complications, and toxoplasmosis. A polypeptide or polynucleotide of the present invention can be used to treat or detect any of these symptoms or diseases.

Preferably, treatment using a polypeptide or polynucleotide of the present invention could either be by administering an effective amount of a polypeptide to the  
25 patient, or by removing cells from the patient, supplying the cells with a polynucleotide of the present invention, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the polypeptide or polynucleotide of the present invention can be used as an antigen in a vaccine to raise an immune response against infectious disease.

30

### **Regeneration**

A polynucleotide or polypeptide of the present invention can be used to differentiate, proliferate, and attract cells, leading to the regeneration of tissues. (See, Science 276:59-87 (1997).) The regeneration of tissues could be used to repair, replace, or protect tissue damaged by congenital defects, trauma (wounds, burns, incisions, or ulcers), age, disease (e.g. osteoporosis, osteoarthritis, periodontal disease, liver failure), surgery, including cosmetic plastic surgery, fibrosis, reperfusion injury, or systemic cytokine damage.

Tissues that could be regenerated using the present invention include organs (e.g., pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac), vasculature (including vascular and lymphatics), nervous, hematopoietic, and skeletal (bone, cartilage, tendon, and ligament) tissue. Preferably, regeneration occurs without or decreased scarring. Regeneration also may include angiogenesis.

Moreover, a polynucleotide or polypeptide of the present invention may increase regeneration of tissues difficult to heal. For example, increased tendon/ligament regeneration would quicken recovery time after damage. A polynucleotide or polypeptide of the present invention could also be used prophylactically in an effort to avoid damage. Specific diseases that could be treated include of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. A further example of tissue regeneration of non-healing wounds includes pressure ulcers, ulcers associated with vascular insufficiency, surgical, and traumatic wounds.

Similarly, nerve and brain tissue could also be regenerated by using a polynucleotide or polypeptide of the present invention to proliferate and differentiate nerve cells. Diseases that could be treated using this method include central and peripheral nervous system diseases, neuropathies, or mechanical and traumatic disorders (e.g., spinal cord disorders, head trauma, cerebrovascular disease, and stroke). Specifically, diseases associated with peripheral nerve injuries, peripheral neuropathy (e.g., resulting from chemotherapy or other medical therapies), localized neuropathies, and central nervous system diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome), could all be treated using the polynucleotide or polypeptide of the present invention.

### Chemotaxis

A polynucleotide or polypeptide of the present invention may have chemotaxis activity. A chemotactic molecule attracts or mobilizes cells (e.g.,  
5 monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells) to a particular site in the body, such as inflammation, infection, or site of hyperproliferation. The mobilized cells can then fight off and/or heal the particular trauma or abnormality.

A polynucleotide or polypeptide of the present invention may increase  
10 chemotactic activity of particular cells. These chemotactic molecules can then be used to treat inflammation, infection, hyperproliferative disorders, or any immune system disorder by increasing the number of cells targeted to a particular location in the body. For example, chemotactic molecules can be used to treat wounds and other trauma to tissues by attracting immune cells to the injured location. Chemotactic  
15 molecules of the present invention can also attract fibroblasts, which can be used to treat wounds.

It is also contemplated that a polynucleotide or polypeptide of the present invention may inhibit chemotactic activity. These molecules could also be used to treat disorders. Thus, a polynucleotide or polypeptide of the present invention could  
20 be used as an inhibitor of chemotaxis.

### Binding Activity

A polypeptide of the present invention may be used to screen for molecules that bind to the polypeptide or for molecules to which the polypeptide binds. The  
25 binding of the polypeptide and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of the polypeptide or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., ligands and receptors), or small molecules.

Preferably, the molecule is closely related to the natural ligand of the  
30 polypeptide, e.g., a fragment of the ligand, or a natural substrate, a ligand, a structural or functional mimetic. (See, Coligan et al., *Current Protocols in Immunology*,

1(2):Chapter 5 (1991).) Similarly, the molecule can be closely related to the natural receptor to which the polypeptide binds, or at least, a fragment of the receptor capable of being bound by the polypeptide (e.g., active site). In either case, the molecule can be rationally designed using known techniques.

5            Preferably, the screening for these molecules involves producing appropriate cells which express the polypeptide, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, *Drosophila*, or *E. coli*. Cells expressing the polypeptide (or cell membrane containing the expressed polypeptide) are then preferably contacted with a test compound potentially  
10            containing the molecule to observe binding, stimulation, or inhibition of activity of either the polypeptide or the molecule.

                 The assay may simply test binding of a candidate compound to the polypeptide, wherein binding is detected by a label, or in an assay involving competition with a labeled competitor. Further, the assay may test whether the  
15            candidate compound results in a signal generated by binding to the polypeptide.

                 Alternatively, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide, measuring polypeptide/molecule  
20            activity or binding, and comparing the polypeptide/molecule activity or binding to a standard.

                 Preferably, an ELISA assay can measure polypeptide level or activity in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure polypeptide level or activity by either binding, directly or  
25            indirectly, to the polypeptide or by competing with the polypeptide for a substrate.

                 All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the polypeptide/molecule. Moreover, the assays can discover agents which  
30            may inhibit or enhance the production of the polypeptide from suitably manipulated cells or tissues.

Therefore, the invention includes a method of identifying compounds which bind to a polypeptide of the invention comprising the steps of: (a) incubating a candidate binding compound with a polypeptide of the invention; and (b) determining if binding has occurred. Moreover, the invention includes a method of identifying  
5 agonists/antagonists comprising the steps of: (a) incubating a candidate compound with a polypeptide of the invention, (b) assaying a biological activity, and (b) determining if a biological activity of the polypeptide has been altered.

#### Antisense And Ribozyme (Antagonists)

10 In specific embodiments, antagonists according to the present invention are nucleic acids corresponding to the sequences contained in SEQ ID NO:1, or the complementary strand thereof, and/or to nucleotide sequences contained in the deposited clone BMP. In one embodiment, antisense sequence is generated internally by the organism, in another embodiment, the antisense sequence is separately  
15 administered (see, for example, O'Connor, *Neurochem.*, 56:560 (1991). Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed for example, in Okano, *Neurochem.*, 56:560 (1991);  
20 Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance, Lee et al., *Nucleic Acids Research*, 6:3073 (1979); Cooney et al., *Science*, 241:456 (1988); and Dervan et al., *Science*, 251:1300 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.  
25 For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense  
30 RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into receptor polypeptide.

In one embodiment, the BMP antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the BMP antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding BMP, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, *Nature*, 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., *Cell*, 22:787-797 (1980), the herpes thymidine promoter (Wagner et al., *Proc. Natl. Acad. Sci. U.S.A.*, 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster, et al., *Nature*, 296:39-42 (1982)), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a apoptosis related gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded BMP antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with a BMP RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the



5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., *Nature*, 372:333-335 (1994). Thus, oligonucleotides complementary to either the 5'- or 3'- non-translated, non-coding regions of BMP shown in Figure 1 could be used in an antisense approach to inhibit translation of endogenous BMP mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3'- or coding region of BMP mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

The polynucleotides of the invention can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger et al., *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556 (1989); Lemaitre et al., *Proc. Natl. Acad. Sci.*, 84:648-652 (1987); PCT Publication NO: WO88/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication NO: WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, *e.g.*, Krol et al., *BioTechniques*, 6:958-976 (1988)) or intercalating agents. (See, *e.g.*, Zon, *Pharm. Res.*, 5:539-549 (1988)). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety

which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, 5 inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5 $\phi$ -methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6- 10 isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

15 The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group including, but not limited 20 to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an a-anomeric oligonucleotide. An a-anomeric oligonucleotide forms specific double-stranded 25 hybrids with complementary RNA in which, contrary to the usual b-units, the strands run parallel to each other (Gautier et al., *Nucl. Acids Res.*, 15:6625-6641 (1987)). The oligonucleotide is a 2 $\phi$ -O-methylribonucleotide (Inoue et al., *Nucl. Acids Res.*, 15:6131-6148 (1987)), or a chimeric RNA-DNA analogue (Inoue et al., *FEBS Lett.* 215:327-330 (1987)).

30 Polynucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are

commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (*Nucl. Acids Res.*, 16:3209 (1988)), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85:7448-7451 (1988)), etc.

While antisense nucleotides complementary to the BMP coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred.

Potential antagonists according to the invention also include catalytic RNA, or a ribozyme (See, e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al, *Science*, 247:1222-1225 (1990)). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy BMP mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, *Nature*, 334:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequence of BMP (Figure 1). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the BMP mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

As in the antisense approach, the ribozymes of the invention can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express BMP *in vivo*. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy

endogenous BMP messages and inhibit translation. Since ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

### **Other Activities**

5           A polypeptide or polynucleotide of the present invention may also increase or decrease the differentiation or proliferation of embryonic stem cells, besides, as discussed above, hematopoietic lineage.

          A polypeptide or polynucleotide of the present invention may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye  
10   color, skin, percentage of adipose tissue, pigmentation, size, and shape (e.g., cosmetic surgery). Similarly, a polypeptide or polynucleotide of the present invention may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

          A polypeptide or polynucleotide of the present invention may be used to  
15   change a mammal's mental state or physical state by influencing biorhythms, circadian rhythms, depression (including depressive disorders), tendency for violence, tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

20           A polypeptide or polynucleotide of the present invention may also be used as a food additive or preservative, such as to increase or decrease storage capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

### **Other Preferred Embodiments**

          Other preferred embodiments of the claimed invention include an isolated nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to a sequence of at least about 50 contiguous nucleotides in the nucleotide sequence of SEQ ID NO:X wherein X is any integer as defined in Table 1.

30           Also preferred is a nucleic acid molecule wherein said sequence of contiguous nucleotides is included in the nucleotide sequence of SEQ ID NO:X in the range of

positions beginning with the nucleotide at about the position of the 5' Nucleotide of the Clone Sequence and ending with the nucleotide at about the position of the 3' Nucleotide of the Clone Sequence as defined for SEQ ID NO:X in Table 1.

Also preferred is an isolated nucleic acid molecule comprising a nucleotide  
5 sequence which is at least 95% identical to a sequence of at least about 150 contiguous nucleotides in the nucleotide sequence of SEQ ID NO:X.

Further preferred is an isolated nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to a sequence of at least about 500 contiguous nucleotides in the nucleotide sequence of SEQ ID NO:X.

10 A further preferred embodiment is an isolated nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to the complete nucleotide sequence of SEQ ID NO:X.

Also preferred is an isolated nucleic acid molecule which hybridizes under stringent hybridization conditions to a nucleic acid molecule, wherein said nucleic  
15 acid molecule which hybridizes does not hybridize under stringent hybridization conditions to a nucleic acid molecule having a nucleotide sequence consisting of only A residues or of only T residues.

Also preferred is a composition of matter comprising a DNA molecule which comprises a human cDNA clone identified by a cDNA Clone Identifier in Table 1,  
20 which DNA molecule is contained in the material deposited with the American Type Culture Collection and given the ATCC Deposit Number shown in Table 1 for said cDNA Clone Identifier.

Also preferred is an isolated nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to a sequence of at least 50 contiguous  
25 nucleotides in the nucleotide sequence of a human cDNA clone identified by a cDNA Clone Identifier in Table 1, which DNA molecule is contained in the deposit given the ATCC Deposit Number shown in Table 1.

Also preferred is an isolated nucleic acid molecule, wherein said sequence of at least 50 contiguous nucleotides is included in the nucleotide sequence of the  
30 complete open reading frame sequence encoded by said human cDNA clone.

Also preferred is an isolated nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to sequence of at least 150 contiguous nucleotides in the nucleotide sequence encoded by said human cDNA containing the sequence of SEQ ID NO:X or contained in the ATCC deposited clones.

5 A further preferred embodiment is an isolated nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to sequence of at least 500 contiguous nucleotides in the nucleotide sequence encoded by said human cDNA clone.

A further preferred embodiment is an isolated nucleic acid molecule  
10 comprising a nucleotide sequence which is at least 95% identical to the complete nucleotide sequence encoded by said human cDNA clone.

A further preferred embodiment is a method for detecting in a biological sample a nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to a sequence of at least 50 contiguous nucleotides in a sequence  
15 selected from the group consisting of: a nucleotide sequence of SEQ ID NO:X wherein X is any integer as defined in Table 1; and a nucleotide sequence encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1; which method comprises a step of comparing a nucleotide sequence of at least one  
20 nucleic acid molecule in said sample with a sequence selected from said group and determining whether the sequence of said nucleic acid molecule in said sample is at least 95% identical to said selected sequence.

Also preferred is the above method wherein said step of comparing sequences comprises determining the extent of nucleic acid hybridization between nucleic acid  
25 molecules in said sample and a nucleic acid molecule comprising said sequence selected from said group. Similarly, also preferred is the above method wherein said step of comparing sequences is performed by comparing the nucleotide sequence determined from a nucleic acid molecule in said sample with said sequence selected from said group. The nucleic acid molecules can comprise DNA molecules or RNA  
30 molecules.

A further preferred embodiment is a method for identifying the species, tissue or cell type of a biological sample which method comprises a step of detecting nucleic acid molecules in said sample, if any, comprising a nucleotide sequence that is at least 95% identical to a sequence of at least 50 contiguous nucleotides in a sequence

5 selected from the group consisting of: a nucleotide sequence of SEQ ID NO:X wherein X is any integer as defined in Table 1; and a nucleotide sequence encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

10 The method for identifying the species, tissue or cell type of a biological sample can comprise a step of detecting nucleic acid molecules comprising a nucleotide sequence in a panel of at least two nucleotide sequences, wherein at least one sequence in said panel is at least 95% identical to a sequence of at least 50 contiguous nucleotides in a sequence selected from said group.

15 Also preferred is a method for diagnosing in a subject a pathological condition associated with abnormal structure or expression of a gene encoding a secreted protein identified in Table 1, which method comprises a step of detecting in a biological sample obtained from said subject nucleic acid molecules, if any, comprising a nucleotide sequence that is at least 95% identical to a sequence of at  
20 least 50 contiguous nucleotides in a sequence selected from the group consisting of: a nucleotide sequence of SEQ ID NO:X wherein X is any integer as defined in Table 1; and a nucleotide sequence encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

25 The method for diagnosing a pathological condition can comprise a step of detecting nucleic acid molecules comprising a nucleotide sequence in a panel of at least two nucleotide sequences, wherein at least one sequence in said panel is at least 95% identical to a sequence of at least 50 contiguous nucleotides in a sequence selected from said group.

30 Also preferred is a composition of matter comprising isolated nucleic acid molecules wherein the nucleotide sequences of said nucleic acid molecules comprise

a panel of at least two nucleotide sequences, wherein at least one sequence in said panel is at least 95% identical to a sequence of at least 50 contiguous nucleotides in a sequence selected from the group consisting of: a nucleotide sequence of SEQ ID NO:X wherein X is any integer as defined in Table 1; and a nucleotide sequence  
5 encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1. The nucleic acid molecules can comprise DNA molecules or RNA molecules.

Also preferred is an isolated polypeptide comprising an amino acid sequence  
10 at least 90% identical to a sequence of at least about 10 contiguous amino acids in the amino acid sequence of SEQ ID NO:Y (wherein Y is any integer as defined in Table 1).

Also preferred is an isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence of at least about 30 contiguous amino acids in the  
15 amino acid sequence of SEQ ID NO:Y.

Further preferred is an isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence of at least about 100 contiguous amino acids in the amino acid sequence of SEQ ID NO:Y.

Further preferred is an isolated polypeptide comprising an amino acid  
20 sequence at least 95% identical to the complete amino acid sequence of SEQ ID NO:Y.

Further preferred is an isolated polypeptide comprising an amino acid sequence at least 90% identical to a sequence of at least about 7 contiguous amino acids in the complete amino acid sequence of a protein encoded by a human cDNA  
25 clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

Also preferred is an isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence of at least about 30 contiguous amino acids in the amino acid sequence of protein encoded by a human cDNA clone identified by a  
30 cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.



Also preferred is an isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence of at least about 100 contiguous amino acids in the amino acid sequence of the protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC

5 Deposit Number shown for said cDNA clone in Table 1.

Also preferred is an isolated polypeptide comprising an amino acid sequence at least 95% identical to the amino acid sequence of the protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

10 Further preferred is an isolated antibody which binds specifically to a polypeptide comprising an amino acid sequence that is at least 90% identical to a sequence of at least 7 contiguous amino acids in a sequence selected from the group consisting of: an amino acid sequence of SEQ ID NO:Y wherein Y is any integer as defined in Table 1; and a complete amino acid sequence of a protein encoded by a  
15 human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

Further preferred is a method for detecting in a biological sample a polypeptide comprising an amino acid sequence which is at least 90% identical to a  
20 sequence of at least 7 contiguous amino acids in a sequence selected from the group consisting of: an amino acid sequence of SEQ ID NO:Y wherein Y is any integer as defined in Table 1; and a complete amino acid sequence of a protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table  
25 1; which method comprises a step of comparing an amino acid sequence of at least one polypeptide molecule in said sample with a sequence selected from said group and determining whether the sequence of said polypeptide molecule in said sample is at least 90% identical to said sequence of at least 7 contiguous amino acids.

Also preferred is the above method wherein said step of comparing an amino  
30 acid sequence of at least one polypeptide molecule in said sample with a sequence selected from said group comprises determining the extent of specific binding of

polypeptides in said sample to an antibody which binds specifically to a polypeptide comprising an amino acid sequence that is at least 90% identical to a sequence of at least 7 contiguous amino acids in a sequence selected from the group consisting of: an amino acid sequence of SEQ ID NO:Y wherein Y is any integer as defined in Table 1; 5 and a complete amino acid sequence of a protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

Also preferred is the above method wherein said step of comparing sequences is performed by comparing the amino acid sequence determined from a polypeptide 10 molecule in said sample with said sequence selected from said group.

Also preferred is a method for identifying the species, tissue or cell type of a biological sample which method comprises a step of detecting polypeptide molecules in said sample, if any, comprising an amino acid sequence that is at least 90% identical to a sequence of at least 7 contiguous amino acids in a sequence selected 15 from the group consisting of: an amino acid sequence of SEQ ID NO:Y wherein Y is any integer as defined in Table 1; and a complete amino acid sequence of a secreted protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

Also preferred is the above method for identifying the species, tissue or cell 20 type of a biological sample, which method comprises a step of detecting polypeptide molecules comprising an amino acid sequence in a panel of at least two amino acid sequences, wherein at least one sequence in said panel is at least 90% identical to a sequence of at least 7 contiguous amino acids in a sequence selected from the above 25 group.

Also preferred is a method for diagnosing in a subject a pathological condition associated with abnormal structure or expression of a gene encoding a secreted protein identified in Table 1, which method comprises a step of detecting in a biological sample obtained from said subject polypeptide molecules comprising an 30 amino acid sequence in a panel of at least two amino acid sequences, wherein at least one sequence in said panel is at least 90% identical to a sequence of at least 7

contiguous amino acids in a sequence selected from the group consisting of: an amino acid sequence of SEQ ID NO:Y wherein Y is any integer as defined in Table 1; and a complete amino acid sequence of a protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the  
5 ATCC Deposit Number shown for said cDNA clone in Table 1.

In any of these methods, the step of detecting said polypeptide molecules includes using an antibody.

Also preferred is an isolated nucleic acid molecule comprising a nucleotide sequence which is at least 95% identical to a nucleotide sequence encoding a  
10 polypeptide wherein said polypeptide comprises an amino acid sequence that is at least 90% identical to a sequence of at least 7 contiguous amino acids in a sequence selected from the group consisting of: an amino acid sequence of SEQ ID NO:Y wherein Y is any integer as defined in Table 1; and a complete amino acid sequence of a protein encoded by a human cDNA clone identified by a cDNA Clone Identifier  
15 in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1.

Also preferred is an isolated nucleic acid molecule, wherein said nucleotide sequence encoding a polypeptide has been optimized for expression of said polypeptide in a prokaryotic host.

Also preferred is an isolated nucleic acid molecule, wherein said polypeptide  
20 comprises an amino acid sequence selected from the group consisting of: an amino acid sequence of SEQ ID NO:Y wherein Y is any integer as defined in Table 1; and a complete amino acid sequence of a protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the  
25 ATCC Deposit Number shown for said cDNA clone in Table 1.

Further preferred is a method of making a recombinant vector comprising inserting any of the above isolated nucleic acid molecule into a vector. Also preferred is the recombinant vector produced by this method. Also preferred is a method of making a recombinant host cell comprising introducing the vector into a host cell, as  
30 well as the recombinant host cell produced by this method.

Also preferred is a method of making an isolated polypeptide comprising culturing this recombinant host cell under conditions such that said polypeptide is expressed and recovering said polypeptide. Also preferred is this method of making an isolated polypeptide, wherein said recombinant host cell is a eukaryotic cell and  
5 said polypeptide is a human protein comprising an amino acid sequence selected from the group consisting of: an amino acid sequence of SEQ ID NO:Y, and an amino acid sequence of a protein encoded by a human cDNA clone identified by a cDNA Clone Identifier in Table 1 and contained in the deposit with the ATCC Deposit Number shown for said cDNA clone in Table 1. The isolated polypeptide produced by this  
10 method is also preferred.

Also preferred is a method of treatment of an individual in need of an increased level of a protein activity, which method comprises administering to such an individual a pharmaceutical composition comprising an amount of an isolated polypeptide, polynucleotide, or antibody of the claimed invention effective to increase  
15 the level of said protein activity in said individual.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

### Examples

#### Example 1: Isolation of a Selected cDNA Clone From the Deposited Sample

Each cDNA clone in a cited ATCC deposit is contained in a plasmid vector.

5 Table 1 identifies the vectors used to construct the cDNA library from which each clone was isolated. In many cases, the vector used to construct the library is a phage vector from which a plasmid has been excised. The table immediately below correlates the related plasmid for each phage vector used in constructing the cDNA library. For example, where a particular clone is identified in Table 1 as being

10 isolated in the vector "Lambda Zap," the corresponding deposited clone is in "pBluescript."

	<u>Vector Used to Construct Library</u>	<u>Corresponding Deposited</u>
	<u>Plasmid</u>	
	Lambda Zap	pBluescript (pBS)
15	Uni-Zap XR	pBluescript (pBS)
	Zap Express	pBK
	lafmid BA	plafmid BA
	pSport1	pSport1
	pCMVSPORT 2.0	pCMVSPORT 2.0
20	pCMVSPORT 3.0	pCMVSPORT 3.0
	pCR <sup>®</sup> 2.1	pCR <sup>®</sup> 2.1

Vectors Lambda Zap (U.S. Patent Nos. 5,128,256 and 5,286,636), Uni-Zap XR (U.S. Patent Nos. 5,128, 256 and 5,286,636), Zap Express (U.S. Patent Nos. 5,128,256 and 5,286,636), pBluescript (pBS) (Short et al., *Nucleic Acids Res.*, 16:7583-7600 (1988); Altling-Mees et al., *Nucleic Acids Res.*, 17:9494 (1989)) and

25 pBK (Altling-Mees et al., *Strategies*, 5:58-61 (1992)) are commercially available from Stratagene Cloning Systems, Inc., 11011 N. Torrey Pines Road, La Jolla, CA, 92037. pBS contains an ampicillin resistance gene and pBK contains a neomycin resistance gene. Both can be transformed into E. coli strain XL-1 Blue, also available

30 from Stratagene. pBS comes in 4 forms SK+, SK-, KS+ and KS-. The S and K refers to the orientation of the polylinker to the T7 and T3 primer sequences which flank the

polylinker region ("S" is for SacI and "K" is for KpnI which are the first sites on each respective end of the linker). "+" or "-" refer to the orientation of the f1 origin of replication ("ori"), such that in one orientation, single stranded rescue initiated from the f1 ori generates sense strand DNA and in the other, antisense.

- 5        Vectors pSport1, pCMVSPORT 2.0 and pCMVSPORT 3.0, were obtained from Life Technologies, Inc., P. O. Box 6009, Gaithersburg, MD 20897. All Sport vectors contain an ampicillin resistance gene and may be transformed into E. coli strain DH10B, also available from Life Technologies. (See, for instance, Gruber, C. E., et al., *Focus* 15:59 (1993).) Vector lafmid BA (Bento Soares, Columbia University, NY) contains an ampicillin resistance gene and can be transformed into E. coli strain 10        XL-1 Blue. Vector pCR<sup>®</sup>2.1, which is available from Invitrogen, 1600 Faraday Avenue, Carlsbad, CA 92008, contains an ampicillin resistance gene and may be transformed into E. coli strain DH10B, available from Life Technologies. (See, for instance, Clark, *Nuc. Acids Res.*, 16:9677-9686 (1988) and Mead et al., 15        *Bio/Technology*, 9 (1991).) Preferably, a polynucleotide of the present invention does not comprise the phage vector sequences identified for the particular clone in Table 1, as well as the corresponding plasmid vector sequences designated above.

      The deposited material in the sample assigned the ATCC Deposit Number cited in Table 1 for any given cDNA clone also may contain one or more additional 20        plasmids, each comprising a cDNA clone different from that given clone. Thus, deposits sharing the same ATCC Deposit Number contain at least a plasmid for each cDNA clone identified in Table 1. Typically, each ATCC deposit sample cited in Table 1 comprises a mixture of approximately equal amounts (by weight) of about 50 plasmid DNAs, each containing a different cDNA clone; but such a deposit sample 25        may include plasmids for more or less than 50 cDNA clones, up to about 500 cDNA clones.

      Two approaches can be used to isolate a particular clone from the deposited sample of plasmid DNAs cited for that clone in Table 1. First, a plasmid is directly isolated by screening the clones using a polynucleotide probe corresponding to SEQ 30        ID NO:X.

Particularly, a specific polynucleotide with 30-40 nucleotides is synthesized using an Applied Biosystems DNA synthesizer according to the sequence reported. The oligonucleotide is labeled, for instance, with  $^{32}\text{P}$ - $\gamma$ -ATP using T4 polynucleotide kinase and purified according to routine methods. (E.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring, NY (1982).)  
5 The plasmid mixture is transformed into a suitable host, as indicated above (such as XL-1 Blue (Stratagene)) using techniques known to those of skill in the art, such as those provided by the vector supplier or in related publications or patents cited above. The transformants are plated on 1.5% agar plates (containing the appropriate selection  
10 agent, e.g., ampicillin) to a density of about 150 transformants (colonies) per plate. These plates are screened using Nylon membranes according to routine methods for bacterial colony screening (e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edit., (1989), Cold Spring Harbor Laboratory Press, pages 1.93 to 1.104), or other techniques known to those of skill in the art.

15 Alternatively, two primers of 17-20 nucleotides derived from both ends of the SEQ ID NO:X (i.e., within the region of SEQ ID NO:X bounded by the 5' NT and the 3' NT of the clone defined in Table 1) are synthesized and used to amplify the desired cDNA using the deposited cDNA plasmid as a template. The polymerase chain reaction is carried out under routine conditions, for instance, in 25  $\mu\text{l}$  of reaction  
20 mixture with 0.5  $\mu\text{g}$  of the above cDNA template. A convenient reaction mixture is 1.5-5 mM  $\text{MgCl}_2$ , 0.01% (w/v) gelatin, 20  $\mu\text{M}$  each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 0.25 Unit of Taq polymerase. Thirty five cycles of PCR (denaturation at 94°C for 1 min; annealing at 55°C for 1 min; elongation at 72°C for 1 min) are performed with a Perkin-Elmer Cetus automated thermal cycler. The  
25 amplified product is analyzed by agarose gel electrophoresis and the DNA band with expected molecular weight is excised and purified. The PCR product is verified to be the selected sequence by subcloning and sequencing the DNA product.

Several methods are available for the identification of the 5' or 3' non-coding portions of a gene which may not be present in the deposited clone. These methods  
30 include but are not limited to, filter probing, clone enrichment using specific probes, and protocols similar or identical to 5' and 3' "RACE" protocols which are well

known in the art. For instance, a method similar to 5' RACE is available for generating the missing 5' end of a desired full-length transcript. (Fromont-Racine et al., *Nucleic Acids Res.*, 21(7):1683-1684 (1993).)

Briefly, a specific RNA oligonucleotide is ligated to the 5' ends of a  
5 population of RNA presumably containing full-length gene RNA transcripts. A primer set containing a primer specific to the ligated RNA oligonucleotide and a primer specific to a known sequence of the gene of interest is used to PCR amplify the 5' portion of the desired full-length gene. This amplified product may then be sequenced and used to generate the full length gene.

10 This above method starts with total RNA isolated from the desired source, although poly-A+ RNA can be used. The RNA preparation can then be treated with phosphatase if necessary to eliminate 5' phosphate groups on degraded or damaged RNA which may interfere with the later RNA ligase step. The phosphatase should then be inactivated and the RNA treated with tobacco acid pyrophosphatase in order  
15 to remove the cap structure present at the 5' ends of messenger RNAs. This reaction leaves a 5' phosphate group at the 5' end of the cap cleaved RNA which can then be ligated to an RNA oligonucleotide using T4 RNA ligase.

This modified RNA preparation is used as a template for first strand cDNA synthesis using a gene specific oligonucleotide. The first strand synthesis reaction is  
20 used as a template for PCR amplification of the desired 5' end using a primer specific to the ligated RNA oligonucleotide and a primer specific to the known sequence of the gene of interest. The resultant product is then sequenced and analyzed to confirm that the 5' end sequence belongs to the desired gene.

## 25 **Example 2: Isolation of Genomic Clones Corresponding to a Polynucleotide**

A human genomic P1 library (Genomic Systems, Inc.) is screened by PCR using primers selected for the cDNA sequence corresponding to SEQ ID NO:X., according to the method described in Example 1. (See also, Sambrook.)

## 30 **Example 3: Tissue Distribution of Polypeptide**



Tissue distribution of mRNA expression of polynucleotides of the present invention is determined using protocols for Northern blot analysis, described by, among others, Sambrook et al. For example, a cDNA probe produced by the method described in Example 1 is labeled with P<sup>32</sup> using the rediprime™ DNA labeling  
5 system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for mRNA expression.

10 Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) (Clontech) are examined with the labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70°C overnight, and the films developed  
15 according to standard procedures.

#### **Example 4: Chromosomal Mapping of the Polynucleotides**

An oligonucleotide primer set is designed according to the sequence at the 5' end of SEQ ID NO:X. This primer preferably spans about 100 nucleotides. This  
20 primer set is then used in a polymerase chain reaction under the following set of conditions : 30 seconds, 95°C; 1 minute, 56°C; 1 minute, 70°C. This cycle is repeated 32 times followed by one 5 minute cycle at 70°C. Human, mouse, and hamster DNA is used as template in addition to a somatic cell hybrid panel containing individual chromosomes or chromosome fragments (Bios, Inc). The reactions is  
25 analyzed on either 8% polyacrylamide gels or 3.5 % agarose gels. Chromosome mapping is determined by the presence of an approximately 100 bp PCR fragment in the particular somatic cell hybrid.

#### **Example 5: Bacterial Expression of a Polypeptide**

30 A polynucleotide encoding a polypeptide of the present invention is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' ends of the DNA

sequence, as outlined in Example 1, to synthesize insertion fragments. The primers used to amplify the cDNA insert should preferably contain restriction sites, such as BamHI and XbaI and initiation/stop codons, if necessary, to clone the amplified product into the expression vector. For example, BamHI and XbaI correspond to the  
5 restriction enzyme sites on the bacterial expression vector pQE-9. (Qiagen, Inc., Chatsworth, CA). This plasmid vector encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter/operator (P/O), a ribosome binding site (RBS), a 6-histidine tag (6-His), and restriction enzyme cloning sites.

10 The pQE-9 vector is digested with BamHI and XbaI and the amplified fragment is ligated into the pQE-9 vector maintaining the reading frame initiated at the bacterial RBS. The ligation mixture is then used to transform the E. coli strain M15/rep4 (Qiagen, Inc.) which contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>).

15 Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml).

20 The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG (Isopropyl-B-D-thiogalacto pyranoside) is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression.

25 Cells are grown for an extra 3 to 4 hours. Cells are then harvested by centrifugation (20 mins at 6000Xg). The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl by stirring for 3-4 hours at 4°C. The cell debris is removed by centrifugation, and the supernatant containing the polypeptide is loaded onto a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (available from  
30 QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the Ni-NTA resin with

high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist (1995) QIAGEN, Inc., *supra*).

Briefly, the supernatant is loaded onto the column in 6 M guanidine-HCl, pH 8, the column is first washed with 10 volumes of 6 M guanidine-HCl, pH 8, then  
5 washed with 10 volumes of 6 M guanidine-HCl pH 6, and finally the polypeptide is eluted with 6 M guanidine-HCl, pH 5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-  
10 NTA column. The recommended conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH 7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins are eluted by the addition of 250 mM imidazole. Imidazole is removed by a final dialyzing step against PBS or 50  
15 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein is stored at 4°C or frozen at -80°C.

In addition to the above expression vector, the present invention further includes an expression vector comprising phage operator and promoter elements operatively linked to a polynucleotide of the present invention, called pHE4a. (ATCC  
20 Accession Number 209645, deposited on February 25, 1998.) This vector contains: 1) a neomycinphosphotransferase gene as a selection marker, 2) an E. coli origin of replication, 3) a T5 phage promoter sequence, 4) two lac operator sequences, 5) a Shine-Delgarno sequence, and 6) the lactose operon repressor gene (*lacIq*). The origin of replication (*oriC*) is derived from pUC19 (LTI, Gaithersburg, MD). The  
25 promoter sequence and operator sequences are made synthetically.

DNA can be inserted into the pHEa by restricting the vector with NdeI and XbaI, BamHI, XhoI, or Asp718, running the restricted product on a gel, and isolating the larger fragment (the stuffer fragment should be about 310 base pairs). The DNA insert is generated according to the PCR protocol described in Example 1, using PCR  
30 primers having restriction sites for NdeI (5' primer) and XbaI, BamHI, XhoI, or

Asp718 (3' primer). The PCR insert is gel purified and restricted with compatible enzymes. The insert and vector are ligated according to standard protocols.

The engineered vector could easily be substituted in the above protocol to express protein in a bacterial system.

5

**Example 6: Purification of a Polypeptide from an Inclusion Body**

The following alternative method can be used to purify a polypeptide expressed in *E. coli* when it is present in the form of inclusion bodies. Unless otherwise specified, all of the following steps are conducted at 4-10°C.

10 Upon completion of the production phase of the *E. coli* fermentation, the cell culture is cooled to 4-10°C and the cells harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution containing 100 mM  
15 Tris, 50 mM EDTA, pH 7.4. The cells are dispersed to a homogeneous suspension using a high shear mixer.

The cells are then lysed by passing the solution through a microfluidizer (Microfluidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by  
20 centrifugation at 7000 xg for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GuHCl) for 2-4 hours. After 7000 xg centrifugation for 15 min., the pellet is discarded and the polypeptide containing supernatant is incubated at 4°C  
25 overnight to allow further GuHCl extraction.

Following high speed centrifugation (30,000 xg) to remove insoluble particles, the GuHCl solubilized protein is refolded by quickly mixing the GuHCl extract with 20 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4°C  
30 without mixing for 12 hours prior to further purification steps.

To clarify the refolded polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16  $\mu\text{m}$  membrane filter with appropriate surface area (e.g., Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perseptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a stepwise manner. The absorbance at 280 nm of the effluent is continuously monitored. Fractions are collected and further analyzed by SDS-PAGE.

Fractions containing the polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perseptive Biosystems) and weak anion (Poros CM-20, Perseptive Biosystems) exchange resins. The columns are equilibrated with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under constant  $A_{280}$  monitoring of the effluent. Fractions containing the polypeptide (determined, for instance, by 16% SDS-PAGE) are then pooled.

The resultant polypeptide should exhibit greater than 95% purity after the above refolding and purification steps. No major contaminant bands should be observed from Commassie blue stained 16% SDS-PAGE gel when 5  $\mu\text{g}$  of purified protein is loaded. The purified protein can also be tested for endotoxin/LPS contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

25

#### **Example 7: Cloning and Expression of a Polypeptide in a Baculovirus Expression System**

In this example, the plasmid shuttle vector pA2 is used to insert a polynucleotide into a baculovirus to express a polypeptide. This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites such as

30

BamHI, Xba I and Asp718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak *Drosophila* promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate a viable virus that express the cloned polynucleotide.

Many other baculovirus vectors can be used in place of the vector above, such as pAc373, pVL941, and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow et al., *Virology* 170:31-39 (1989).

Specifically, the cDNA sequence contained in the deposited clone is amplified using the PCR protocol described in Example 1 using primers with appropriate restriction sites and initiation/stop codons. If the naturally occurring signal sequence is used to produce the secreted protein, the pA2 vector does not need a second signal peptide. Alternatively, the vector can be modified (pA2 GP) to include a baculovirus leader sequence, using the standard methods described in Summers et al., "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures," Texas Agricultural Experimental Station Bulletin NO: 1555 (1987).

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

The plasmid is digested with the corresponding restriction enzymes and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.).

The fragment and the dephosphorylated plasmid are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue

(Stratagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria containing the plasmid are identified by digesting DNA from individual colonies and analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing.

Five  $\mu\text{g}$  of a plasmid containing the polynucleotide is co-transfected with 1.0  $\mu\text{g}$  of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA), using the lipofection method described by Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7417 (1987). One  $\mu\text{g}$  of BaculoGold™ virus DNA and 5  $\mu\text{g}$  of the plasmid are mixed in a sterile well of a microtiter plate containing 50  $\mu\text{l}$  of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10  $\mu\text{l}$  Lipofectin plus 90  $\mu\text{l}$  Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is then incubated for 5 hours at 27° C. The transfection solution is then removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. Cultivation is then continued at 27° C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, *supra*. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10.) After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200  $\mu\text{l}$  of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4° C.

To verify the expression of the polypeptide, Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus containing the polynucleotide at a multiplicity of infection ("MOI") of about 2. If radiolabeled proteins are desired, 6 hours later the  
5 medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). After 42 hours, 5  $\mu\text{Ci}$  of  $^{35}\text{S}$ -methionine and 5  $\mu\text{Ci}$   $^{35}\text{S}$ -cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by  
10 SDS-PAGE followed by autoradiography (if radiolabeled).

Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the produced protein.

#### **Example 8: Expression of a Polypeptide in Mammalian Cells**

15 The polypeptide of the present invention can be expressed in a mammalian cell. A typical mammalian expression vector contains a promoter element, which mediates the initiation of transcription of mRNA, a protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening  
20 sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription is achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLV, HIV and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

25 Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), pCMVSPORT 2.0, and pCMVSPORT 3.0. Mammalian host cells that could be used include, human HeLa, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1,



Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the polypeptide can be expressed in stable cell lines containing the polynucleotide integrated into a chromosome. The co-transfection with a  
5 selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful in developing cell lines that carry several hundred or even several thousand copies of the gene of  
10 interest. (See, e.g., Alt et al., *J. Biol. Chem.*, 253:1357-1370 (1978); Hamlin et al., *Biochem. et Biophys. Acta*, 1097:107-143 (1990); Page et al., *Biotechnology*, 9:64-68 (1991)). Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., *Biochem J.*, 227:277-279 (1991); Bebbington et al., *Bio/Technology*, 10:169-175 (1992). Using these markers, the mammalian cells are grown in selective  
15 medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

Derivatives of the plasmid pSV2-dhfr (ATCC Accession No.: 37146), the expression vectors pC4 (ATCC Accession No.: 209646) and pC6 (ATCC Accession  
20 No.:209647) contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., *Molecular and Cellular Biology*, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., *Cell*, 41:521-530 (1985).) Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors also contain the 3' intron, the  
25 polyadenylation and termination signal of the rat preproinsulin gene, and the mouse DHFR gene under control of the SV40 early promoter.

Specifically, the plasmid pC6, for example, is digested with appropriate restriction enzymes and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

30 A polynucleotide of the present invention is amplified according to the protocol outlined in Example 1 using primers with appropriate restrictions sites and

initiation/stop codons, if necessary. The vector can be modified to include a heterologous signal sequence if necessary for secretion. (See, e.g., WO 96/34891.)

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment  
5 then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

The amplified fragment is then digested with the same restriction enzyme and purified on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then  
10 transformed and bacteria are identified that contain the fragment inserted into plasmid pC6 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene is used for transfection. Five  $\mu$ g of the expression plasmid pC6 is cotransfected with 0.5  $\mu$ g of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid pSV2-neo  
15 contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml  
20 G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1  $\mu$ M, 2  $\mu$ M, 5  $\mu$ M, 10 mM, 20 mM). The same  
25 procedure is repeated until clones are obtained which grow at a concentration of 100 - 200  $\mu$ M. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

#### **Example 9: Protein Fusions**

30 The polypeptides of the present invention are preferably fused to other proteins. These fusion proteins can be used for a variety of applications. For

example, fusion of the present polypeptides to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See Example 5; see also EP A 394,827; Traunecker, et al., *Nature*, 331:84-86 (1988)) The polypeptides can also be fused to heterologous polypeptide sequences to facilitate secretion and intracellular trafficking (e.g., KDEL). Moreover, fusion to IgG-1, IgG-3, and albumin increases the half-life time in vivo. Nuclear localization signals fused to the polypeptides of the present invention can target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of a fusion protein. Fusion proteins can also create chimeric molecules having more than one function. Finally, fusion proteins can increase solubility and/or stability of the fused protein compared to the non-fused protein. All of the types of fusion proteins described above can be made by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule, or the protocol described in Example 5.

Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian expression vector, and initiation/stop codons, if necessary.

For example, if pC4 (Accession No.: 209646) is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and a polynucleotide of the present invention, isolated by the PCR protocol described in Example 1, is ligated into this BamHI site. Note that the polynucleotide is cloned without a stop codon, otherwise a fusion protein will not be produced.

If the naturally occurring signal sequence is used to produce the secreted protein, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., WO 96/34891.)

**Human IgG Fc region:**

GGGATCCGGAGCCCAAATCTTCTGACAAAACCTCACACATGCCACCGTGCCAGCACCTGAATTCGAGG  
 GTGCACCGTCAGTCTTCTCTTCCCCCAAACCAAGGACACCCATCATGATCTCCCGGACTCCTGAGG  
 TCACATGCGTGGTGGTGGACGTAAGCCACGAAGACCCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCG  
 5 TGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCG  
 TCCTCACCGTCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCC  
 TCCCAACCCCATCGAGAAAACCATCTCCAAAGCCAAAGGCAGCCCCGAGAACCACAGGTGTACACCC  
 TGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATC  
 CAAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGAGACAACACTACAAGACCACGCCTCCCG  
 10 TGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGG  
 GGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCC  
 TGTCTCCGGGTAAATGAGTGCGACGGCCGCGACTCTAGAGGAT (SEQ ID NO:1)

**Example 10: Formulating a Polypeptide**

15 The polypeptide composition will be formulated and dosed in a fashion  
 consistent with good medical practice, taking into account the clinical condition of the  
 individual patient (especially the side effects of treatment with the secreted  
 polypeptide alone), the site of delivery, the method of administration, the scheduling  
 of administration, and other factors known to practitioners. The "effective amount"  
 20 for purposes herein is thus determined by such considerations.

As a general proposition, the total pharmaceutically effective amount of  
 polypeptide administered parenterally per dose will be in the range of about 1  
 $\mu\text{g/kg/day}$  to 10  $\text{mg/kg/day}$  of patient body weight, although, as noted above, this will  
 be subject to therapeutic discretion. More preferably, this dose is at least 0.01  
 25  $\text{mg/kg/day}$ , and most preferably for humans between about 0.01 and 1  $\text{mg/kg/day}$  for  
 the hormone. If given continuously, the polypeptide is typically administered at a  
 dose rate of about 1  $\mu\text{g/kg/hour}$  to about 50  $\mu\text{g/kg/hour}$ , either by 1-4 injections per  
 day or by continuous subcutaneous infusions, for example, using a mini-pump. An  
 intravenous bag solution may also be employed. The length of treatment needed to  
 30 observe changes and the interval following treatment for responses to occur appears  
 to vary depending on the desired effect.

Pharmaceutical compositions containing the polypeptide of the invention are  
 administered orally, rectally, parenterally, intracisternally, intravaginally,

intraperitoneally, topically (as by powders, ointments, gels, drops or transdermal patch), buccally, or as an oral or nasal spray. "Pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to  
5 modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

The polypeptide is also suitably administered by sustained-release systems. Suitable examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules. Sustained-release  
10 matrices include polylactides (U.S. Pat. NO: 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman et al., *Biopolymers*, 22:547-556 (1983)), poly (2- hydroxyethyl methacrylate) (Langer et al., *J. Biomed. Mater. Res.* 15:167-277 (1981), and Langer, *Chem. Tech.*, 12:98-105 (1982)), ethylene vinyl acetate (R. Langer et al.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988).  
15 Sustained-release compositions also include liposomally entrapped polypeptides. Liposomes containing the secreted polypeptide are prepared by methods known per se: DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688-3692 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S.  
20 Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal secreted polypeptide therapy.

For parenteral administration, in one embodiment, the polypeptide is  
25 formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other  
30 compounds that are known to be deleterious to polypeptides.

Generally, the formulations are prepared by contacting the polypeptide uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood  
5 of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to  
10 recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as  
15 polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, manose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

20 The polypeptide is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of polypeptide salts.

Any polypeptide to be used for therapeutic administration can be sterile.  
25 Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutic polypeptide compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Polypeptides ordinarily will be stored in unit or multi-dose containers, for  
30 example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml

vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous polypeptide solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized polypeptide using bacteriostatic Water-for-Injection.

The invention also provides a pharmaceutical pack or kit comprising one or  
5 more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the  
10 polypeptides of the present invention may be employed in conjunction with other therapeutic compounds.

#### **Example 11: Method of Treating Decreased Levels of the Polypeptide**

It will be appreciated that conditions caused by a decrease in the standard or  
15 normal expression level of a polypeptide in an individual can be treated by administering the polypeptide of the present invention, preferably in the secreted and/or soluble form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of the polypeptide comprising administering to such an individual a pharmaceutical composition comprising an amount of the  
20 polypeptide to increase the activity level of the polypeptide in such an individual.

For example, a patient with decreased levels of a polypeptide receives a daily dose 0.1-100 ug/kg of the polypeptide for six consecutive days. Preferably, the polypeptide is in the secreted form. The exact details of the dosing scheme, based on administration and formulation, are provided in Example 10.

25

#### **Example 12: Method of Treating Increased Levels of the Polypeptide**

Antisense technology is used to inhibit production of a polypeptide of the present invention. This technology is one example of a method of decreasing levels of a polypeptide, preferably a secreted form, due to a variety of etiologies, such as  
30 cancer.

For example, a patient diagnosed with abnormally increased levels of a polypeptide is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the treatment was well tolerated. The formulation of the antisense polynucleotide is provided in Example 10.

**Example 13: Method of Treatment Using Gene Therapy - Ex Vivo**

One method of gene therapy transplants fibroblasts, which are capable of expressing a polypeptide, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37°C for approximately one week.

At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention can be amplified using PCR primers which correspond to the 5' and 3' end sequences respectively as set forth in Example 1 using primers and having appropriate restriction sites and initiation/stop codons, if necessary. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is



maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector has the gene of interest properly inserted.

5       The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the  
10       packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is  
15       removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been  
20       efficiently infected, the fibroblasts are analyzed to determine whether protein is produced.

The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

#### 25       Example 14: Gene Therapy Using Endogenous BMP Genes

Another method of gene therapy according to the present invention involves operably associating the endogenous BMP sequence with a promoter via homologous recombination as described, for example, in U.S. Patent NO: 5,641,670, issued June 24, 1997; International Publication NO: WO 96/29411, published September 26,  
30       1996; International Publication NO: WO 94/12650, published August 4, 1994; Koller et al., *Proc. Natl. Acad. Sci. USA*, 86:8932-8935 (1989); and Zijlstra et al., *Nature*,

342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not expressed in the cells, or is expressed at a lower level than desired.

Polynucleotide constructs are made which contain a promoter and targeting  
5 sequences, which are homologous to the 5' non-coding sequence of endogenous BMP,  
flanking the promoter. The targeting sequence will be sufficiently near the 5' end of  
BMP so the promoter will be operably linked to the endogenous sequence upon  
homologous recombination. The promoter and the targeting sequences can be  
amplified using PCR. Preferably, the amplified promoter contains distinct restriction  
10 enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting  
sequence contains the same restriction enzyme site as the 5' end of the amplified  
promoter and the 5' end of the second targeting sequence contains the same restriction  
site as the 3' end of the amplified promoter.

The amplified promoter and the amplified targeting sequences are digested  
15 with the appropriate restriction enzymes and subsequently treated with calf intestinal  
phosphatase. The digested promoter and digested targeting sequences are added  
together in the presence of T4 DNA ligase. The resulting mixture is maintained under  
conditions appropriate for ligation of the two fragments. The construct is size  
fractionated on an agarose gel then purified by phenol extraction and ethanol  
20 precipitation.

In this Example, the polynucleotide constructs are administered as naked  
polynucleotides via electroporation. However, the polynucleotide constructs may  
also be administered with transfection-facilitating agents, such as liposomes, viral  
sequences, viral particles, precipitating agents, etc. Such methods of delivery are  
25 known in the art.

Once the cells are transfected, homologous recombination will take place  
which results in the promoter being operably linked to the endogenous BMP  
sequence. This results in the expression of BMP in the cell. Expression may be  
detected by immunological staining, or any other method known in the art.

30 Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is  
placed in DMEM + 10% fetal calf serum. Exponentially growing or early stationary

phase fibroblasts are trypsinized and rinsed from the plastic surface with nutrient medium. An aliquot of the cell suspension is removed for counting, and the remaining cells are subjected to centrifugation. The supernatant is aspirated and the pellet is resuspended in 5 ml of electroporation buffer (20 mM HEPES pH 7.3, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub> HPO<sub>4</sub>, 6 mM dextrose). The cells are recentrifuged, the supernatant aspirated, and the cells resuspended in electroporation buffer containing 1 mg/ml acetylated bovine serum albumin. The final cell suspension contains approximately 3X10<sup>6</sup> cells/ml. Electroporation should be performed immediately following resuspension.

10 Plasmid DNA is prepared according to standard techniques. For example, to construct a plasmid for targeting to the BMP locus, plasmid pUC18 (MBI Fermentas, Amherst, NY) is digested with HindIII. The CMV promoter is amplified by PCR with an XbaI site on the 5' end and a BamHI site on the 3' end. Two BMP non-coding sequences are amplified via PCR: one BMP non-coding sequence (BMP fragment 1) is amplified with a HindIII site at the 5' end and an Xba site at the 3' end; the other BMP non-coding sequence (BMP fragment 2) is amplified with a BamHI site at the 5' end and a HindIII site at the 3' end. The CMV promoter and BMP fragments are digested with the appropriate enzymes (CMV promoter - XbaI and BamHI; BMP fragment 1 - XbaI; BMP fragment 2 - BamHI) and ligated together. The resulting ligation product is digested with HindIII, and ligated with the HindIII-digested pUC18 plasmid.

Plasmid DNA is added to a sterile cuvette with a 0.4 cm electrode gap (Bio-Rad). The final DNA concentration is generally at least 120 µg/ml. 0.5 ml of the cell suspension (containing approximately 1.5X10<sup>6</sup> cells) is then added to the cuvette, and the cell suspension and DNA solutions are gently mixed. Electroporation is performed with a Gene-Pulser apparatus (Bio-Rad). Capacitance and voltage are set at 960 µF and 250-300 V, respectively. As voltage increases, cell survival decreases, but the percentage of surviving cells that stably incorporate the introduced DNA into their genome increases dramatically. Given these parameters, a pulse time of approximately 14-20 mSec should be observed.

Electroporated cells are maintained at room temperature for approximately 5 min, and the contents of the cuvette are then gently removed with a sterile transfer pipette. The cells are added directly to 10 ml of prewarmed nutrient media (DMEM with 15% calf serum) in a 10 cm dish and incubated at 37 degree C. The following  
5 day, the media is aspirated and replaced with 10 ml of fresh media and incubated for a further 16-24 hours.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product. The fibroblasts can then be introduced into a  
10 patient as described above.

**Example 15: Method of Treatment Using Gene Therapy - In Vivo**

Another aspect of the present invention is using *in vivo* gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the  
15 introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) BMP sequences into an animal to increase or decrease the expression of the BMP polypeptide. The BMP polynucleotide may be operatively linked to a promoter or any other genetic elements necessary for the expression of the BMP polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known  
20 in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO: 5693622, 5705151, 5580859; Tabata et al., *Cardiovasc. Res.* 35(3):470-479 (1997), Chao J et al., *Pharmacol. Res.*, 35(6):517-522 (1997), Wolff, *Neuromuscul. Disord.* 7(5):314-318 (1997), Schwartz et al., *Gene Ther.*, 3(5):405-411 (1996), Tsurumi Y. et al., *Circulation*, 94(12):3281-3290 (1996) (incorporated herein by reference).

25 The BMP polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The BMP polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

30 The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the

cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the BMP polynucleotides may also be delivered in liposome formulations (such as those taught in Felgner et al., *Ann. NY Acad. Sci.*, 772:126-139 (1995) and Abdallah et al., *Biol. Cell*, 85(1):1-7 (1995))

5 which can be prepared by methods well known to those skilled in the art.

The BMP polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene  
10 therapeutics techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

15 The polynucleotide constructs can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid,  
20 mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred  
25 for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In*  
30 *vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked BMP polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked BMP polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected BMP polynucleotide in muscle *in vivo* is determined as follows. Suitable BMP template DNA for production of mRNA coding for BMP polypeptide is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The BMP template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for BMP protein expression. A time course for BMP protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of

BMP DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be use to extrapolate proper dosages and other treatment parameters in humans and other  
5 animals using BMP naked DNA.

#### **Example 16: Full Thickness Articular Cartilage Repair Model**

A full thickness articular cartilage defect model in the femoral-patellar joint of  
10 adult rabbits is used to evaluate the ability of the combination of BMPs to affect cartilage and bone repair. Adult New Zealand White rabbits are anesthetized and prepared for sterile surgery. A 3.3 mm defect through articular cartilage and into underlying subchondral bone is drilled into the patellar groove of the knee joint. The defect is either left empty, filled with collagen sponge (controls), or with collagen  
15 sponge soaked with 10 µg BMP. The incision is closed and animals are allowed free movement within their cages for 4 weeks. After 4 weeks the animals are humanely euthanatized and the articular cartilage/subchondral bone defect is evaluated histologically for tissue architecture, quantity and quality of repair tissue. Northern analysis is performed for additional phenotyping.

20

#### **Example 17: Rat Model Bioassay for Tendon/Ligament-Like Tissue Formation**

A modified version of the rat ectopic implant assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. USA, 80:6591-6595 (1983) is another method used to evaluate the activity of the BMPs. This modified assay is herein called the Rosen-  
25 modified Sampath-Reddi assay. The assay has been widely used to evaluate the bone and cartilage-inducing activity of BMPs. The ethanol precipitation step of the Sampath-Reddi procedure is replaced by dialyzing (if the composition is a solution) or diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then equilibrated to 0.1% TFA. The resulting  
30 solution is added to 20 mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted

subcutaneously in the abdominal thoracic area of 21-49 day old male Long Evans rats. The implants are removed after 10 days. A section of each implant is fixed and processed for histological analysis. One (1)  $\mu$ m glycolmethacrylate sections are stained with Von Kossa and acid fuchsin to score the amount of induced tendon/ligament-like tissue formation present in each implant.

#### **Example 18: Rat Model Bioassay for Bone Induction**

This assay consists of implanting allogenic or xenogenic test samples in subcutaneous sites in recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, may be used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the BMP test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants are removed on day 12. The heterotopic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotopic sites.

Bone inducing activity is determined biochemically by the specific activity of alkaline phosphatase and calcium content of the day 12 implant. An increase in the specific activity of alkaline phosphatase indicates the onset of bone formation. Calcium content, on the other hand, is proportional to the amount of bone formed in the implant. Bone formation therefore is calculated by determining the calcium content of the implant on day 12 in rats and is expressed as "bone forming units," where one bone forming unit represents the amount of protein that is needed for half maximal bone forming activity of the implant on day 12. Bone induction exhibited by intact demineralized rat bone matrix is considered to be the maximal bone differentiation activity for comparison purposes in this assay.

Successful implants exhibit a controlled progression through the stages of protein-induced endochondral bone development, including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight;



(6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoclasts, bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicles on day twenty-one. It is possible that increasing  
5 amounts of one or more BMPs may accelerate this time course. The shape of the new bone conforms to the shape of the implanted matrix.

Histological sectioning and staining is preferred to determine the extent of osteogenesis in the implants. Implants are fixed in Bouins Solution, embedded in paraffin, and cut into 6-8  $\mu\text{m}$  sections. Staining with toluidine blue or  
10 hemotoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve-day implants are usually sufficient to determine whether the implants contain newly-induced bone.

Alkaline phosphatase (AP) activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization  
15 of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantification and obtaining an estimate of bone formation quickly after the implants are removed from the rat. Additionally, alkaline phosphatase activity can be  
20 determined using the W-20 Alkaline Phosphatase Assay Protocol disclosed in International Publication No. WO 99/29718, which is herein incorporated by reference in its entirety. Alternatively, the amount of bone formation can be determined by measuring the calcium content of the implant.

Gene expression patterns that correlate with endochondral bone or other  
25 types of tissue formation can also be monitored by quantitating mRNA levels using procedures known to those of skill in the art such as Northern Blot analysis. Such developmental gene expression markers may be used to determine progression through tissue differentiation pathways after BMP treatments. These markers include osteoblastic-related matrix proteins such as procollagen  $\alpha_2$  (I), procollagen  $\alpha_1$  (I),  
30 procollagen  $\alpha_1$  (III), osteonectin, osteopontin, biglycan, and alkaline phosphatase for

bone regeneration (see, e.g., Suva et al., *J. Bone Miner. Res.*, 8:379-88 (1993); Benayahu et al., *J. Cell. Biochem.*, 56:62-73 (1994)).

**Example 19: Feline Model Bioassay for Bone Repair**

5           A femoral osteotomy defect is surgically prepared. Without further intervention, the simulated fracture defect would consistently progress to non-union. The effects of BMP compositions and devices implanted into the created bone defects are evaluated by the following study protocol.

          The 1 cm and 2 cm femoral defect cat studies demonstrate that devices  
10   comprising a matrix containing a BMP can: (1) repair a weight-bearing bone defect in a large animal; (2) consistently induce bone formation shortly following (less than two weeks) implantation; and (3) induce bone by endochondral ossification, with a strength equal to normal bone, on a volume for volume basis. Furthermore, all animals remain  
15   healthy during the study and show no evidence of clinical or histological laboratory reaction to the implanted device. In this bone defect model, there is little or no healing at control bone implant sites. The results provide evidence for the successful use of the BMP compositions and devices of this invention to repair large, non-union bone defects.

20           Briefly, the procedure is as follows: Sixteen adult cats each weighing less than 10 lbs. undergo unilateral preparation of a 1 cm bone defect in the right femur through a lateral surgical approach. In other experiments, a 2 cm bone defect may be created. The femur is immediately internally fixed by lateral placement of an 8-hole plate to preserve the exact dimensions of the defect.

25           Three different types of materials may be implanted in the surgically created cat femoral defects: group I is a negative control group which undergoes the same plate fixation with implants of 4M guanidine-HCl-treated (inactivated) cat demineralized bone matrix powder (GuHCl-DBM) (360 mg); group II is a positive control group implanted with biologically active demineralized bone matrix powder  
30   (DBM) (360 mg); and groups III and IV undergo a procedure identical to groups I-II, with the addition of a BMP alone (group III) and a combination of more than one

BMP or a BMP and another appropriate factor (group IV) onto each of the GuHCl-DBM carrier samples.

All animals are allowed to ambulate ad libitum within their cages post-operatively. All cats are injected with tetracycline (25 mg/kg subcutaneously (SQ) each week for four weeks) for bone labeling. All but four group III and four group IV animals are sacrificed four months after femoral osteotomy.

In vivo radiomorphometric studies are carried out immediately post-op at 4, 8, 12 and 16 weeks by taking a standardized X-ray of the lightly-anesthetized animal positioned in a cushioned X-ray jig designed to consistently produce a true anterior-posterior view of the femur and the osteotomy site. All X-rays are taken in exactly the same fashion and in exactly the same position on each animal. Bone repair is calculated as a function of mineralization by means of random point analysis. A final specimen radiographic study of the excised bone is taken in two planes after sacrifice.

At 16 weeks, the percentage of groups III and IV femurs that are united, and the average percent bone defect regeneration in groups I-IV are compared. The group I GuHCl-DBM negative-control implants should generally exhibit no bone growth at four weeks, less than 10% at eight and 12 weeks, and about 16% (+/-10%) at 16 weeks. The group II DBM positive-control implants should generally exhibit about 15-20% repair at four weeks, 35% at eight weeks, 50% (+/-10%,) at 12 weeks and 70% (+/-12%) by 16 weeks.

Excised test and normal femurs may be immediately studied by bone densitometry, or wrapped in two layers of saline-soaked towels, placed into sealed plastic bags, and stored at -20°C. until further study. Bone repair strength, load-to-failure, and work-to-failure are tested by loading to failure on a specially designed steel 4-point bending jig attached to an Instron testing machine to quantitate bone strength, stiffness, energy absorbed and deformation to failure. The study of test femurs and normal femurs yields the bone strength (load) in pounds and work-to-failure in joules. Normal femurs exhibit a strength of 96 (+/-12) pounds. BMP device-implanted femur strength should be corrected for surface area at the site of fracture (due to the "hourglass" shape of the bone defect repair). With this correction, the result should correlate closely with normal bone strength.

Following biomechanical testing, the bones are immediately sliced into two longitudinal sections at the defect site, weighed, and the volume measured. One-half is fixed for standard calcified bone histomorphometrics with fluorescent stain incorporation evaluation, and one-half is fixed for decalcified hemotoxylin/eosin stain histology preparation.

Selected specimens from the bone repair site are homogenized in cold 0.15M NaCl, 3 mM NaHCO<sub>3</sub>, pH 9.0 by a Spex freezer mill. The alkaline phosphatase activity of the supernatant and total calcium content of the acid soluble fraction of sediment are then determined.

#### **Example 20: Dog Ulnar Defect Bioassay For Bone Repair**

This assay is performed essentially as described in Cook et al., *Clinical Orthopaedics and Related Research*, 301:302-112 (1994), which is incorporated herein by reference). Briefly, an ulnar segmental defect model is used to evaluate bone healing in 35-45 kg adult male dogs. Experimental composites comprising 500 mg of insoluble bovine bone collagen are reconstituted with either 0, 625, 1200 or 2500 µg of BMP in the absence or presence of increasing concentrations of one or more additional BMPs of the present invention or other factor. Implantations at defect sites are performed with one carrier control and with the experimental series of BMP concentrations being tested. Mechanical testing is performed on ulnae of animals receiving composites at 12 weeks after implantation. Radiographs of the forelimbs are obtained weekly until the animals are sacrificed at either 12 or 16 postoperative weeks. Histological sections are analyzed from the defect site and from adjacent normal bone.

#### **Example 21: Monkey Ulnar and Tibial Defect Bioassay For Bone Repair**

This bone healing assay in African green monkeys is performed essentially as described in Cook et al., *J. Bone and Joint Surgery*, 77A:734-50 (1995), which is incorporated herein by reference. Briefly, a 2.0 cm osteoperiosteal defect is created in the middle of the ulnar shaft and filled with an implant comprising various matrices containing 1000 µg of BMP in the absence or presence of increasing concentrations

of one or more BMPs of the present invention or other factor. Experimental composites comprising various matrices reconstituted with either 0, 250, 500 or 100 or 2000 µg of BMP is used to fill 2.0 cm BMP defects created in the diaphysis of the tibia. Implantations at defect sites are performed with one carrier control and with the experimental series of BMP concentrations being tested. Mechanical testing is performed on ulnae and tibia of animals receiving composites. Radiographs and histological sections are analyzed from the defect sites and from adjacent normal bone as described in Cook et al.

10 **Example 22: Rat Model Bioassay for Nerve Regeneration and Repair**

A matrix carrier is prepared. Wang et al. (WO 95/05846) used Collastat.RTM., a collagen sponge (Vitaphore Wound Healing, Inc.), but any other desired carrier, such as those described herein, may be tested for applicability. The collagen carrier is prepared by washing, lyophilizing, sterilizing and degassing, and is then loaded with, for example, either: with no BMP (negative control group), with BMP only (group I), or with a particular combination of BMPs or BMP(s) and other factor(s) (group II). Variations on the experimental design allow one skilled in the art to test a variety of different BMP combinations under various conditions.

All manipulations are performed under sterile conditions. The loaded matrices are placed inside approximately 1.6 X 20 mm lengths of sterile vented silastic or biodegradable tubing (stents) which may be trimmed to remove excess tubing before surgery. Vented silastic or biodegradable stents containing the matrices are applied microscopically and anastomized to the severed nerve endings, which are inserted into the stent for about 1 mm at each end, leaving a 15 mm "nerve defect" gap. Rats are tested for electrical return of function over a time course of weeks after implantation. Compound muscle action potentials (CMAPs) provide a reproducible transcutaneous measurement for assessing the degree of functional return. CMAP amplitude and latency is proportional to the number of reinnervated axon/motor endplates and thus serves as a useful index of neuronal regeneration.

Animals may be sacrificed for histopathological examination at various times post-implantation. Control stents implanted within subcutaneous tissues serve as histochemical controls.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous  
5 modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other  
10 disclosures) in the Background of the Invention, Detailed Description, and Examples is hereby incorporated herein by reference. Further, the hard copy of the sequence listing submitted herewith and the corresponding computer readable form are both incorporated herein by reference in their entireties.

15 **Example 23: BMP Polypeptide Biological Effects**

**Fibroblast and endothelial cell assays.** Human lung fibroblasts are obtained from Clonetics (San Diego, CA) and maintained in growth media from Clonetics. Dermal microvascular endothelial cells are obtained from Cell Applications (San Diego, CA). For proliferation assays, the human lung fibroblasts and dermal  
20 microvascular endothelial cells can be cultured at 5,000 cells/well in a 96-well plate for one day in growth medium. The cells are then incubated for one day in 0.1% BSA basal medium. After replacing the medium with fresh 0.1% BSA medium, the cells are incubated with the test proteins for 3 days. Alamar Blue (Alamar Biosciences, Sacramento, CA) is added to each well to a final concentration of 10%. The cells are  
25 incubated for 4 hr. Cell viability is measured by reading in a CytoFluor fluorescence reader. For the PGE<sub>2</sub> assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or BMP polypeptide with or without IL-1 $\alpha$  for 24 hours. The supernatants are collected and assayed for PGE<sub>2</sub> by EIA kit  
30 (Cayman, Ann Arbor, MI). For the IL-6 assays, the human lung fibroblasts are

cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or the BMP polypeptide with or without IL-1 $\alpha$  for 24 hours. The supernatants are collected and assayed for IL-6 by ELISA kit (Endogen, Cambridge, MA).

- 5 Human lung fibroblasts are cultured with FGF-2 or the BMP polypeptide for 3 days in basal medium before the addition of Alamar Blue to assess effects on growth of the fibroblasts. FGF-2 should show a stimulation at 10 - 2500 ng/ml which can be used to compare stimulation with the BMP polypeptide.

10 **Example 24: The Effect of the BMP Polypeptide on the Growth of Vascular Endothelial Cells**

- On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at 2-5x10<sup>4</sup> cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnology, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin, BMP protein of, and positive controls, such as basic FGF (bFGF) are added, at varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

- An increase in the number of HUVEC cells indicates that the BMP polypeptide may proliferate vascular endothelial cells.

- The studies described in this example test activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

25 **Example 25: Stimulatory Effect of the BMP Polypeptide on the Proliferation of Vascular Endothelial Cells**

- For evaluation of mitogenic activity of growth factors, the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) assay with the electron coupling reagent PMS (phenazine methosulfate) was performed (CellTiter 96 AQ, Promega). Cells are seeded in a 96-well plate

(5,000 cells/well) in 0.1 mL serum-supplemented medium and are allowed to attach overnight. After serum-starvation for 12 hours in 0.5% FBS, conditions (bFGF, VEGF<sub>165</sub> or the BMP polypeptide in 0.5% FBS) with or without Heparin (8 U/ml) are added to wells for 48 hours. 20 mg of MTS/PMS mixture (1:0.05) are added per well  
5 and allowed to incubate for 1 hour at 37°C before measuring the absorbance at 490 nm in an ELISA plate reader. Background absorbance from control wells (some media, no cells) is subtracted, and seven wells are performed in parallel for each condition. See, Leak *et al. In Vitro Cell. Dev. Biol.* 30A:512-518 (1994).

The studies described in this example tested activity in the BMP protein.  
10 However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

#### **Example 26: Inhibition of PDGF-induced Vascular Smooth Muscle Cell**

##### **15 Proliferation Stimulatory Effect**

HAoSMC proliferation can be measured, for example, by BrdUrd incorporation. Briefly, subconfluent, quiescent cells grown on the 4-chamber slides are transfected with CRP or FITC-labeled AT2-3LP. Then, the cells are pulsed with 10% calf serum and 6 mg/ml BrdUrd. After 24 h, immunocytochemistry is performed  
20 by using BrdUrd Staining Kit (Zymed Laboratories). In brief, the cells are incubated with the biotinylated mouse anti-BrdUrd antibody at 4 °C for 2 h after being exposed to denaturing solution and then incubated with the streptavidin-peroxidase and diaminobenzidine.

After counterstaining with hematoxylin, the cells are mounted for  
25 microscopic examination, and the BrdUrd-positive cells are counted. The BrdUrd index is calculated as a percent of the BrdUrd-positive cells to the total cell number. In addition, the simultaneous detection of the BrdUrd staining (nucleus) and the FITC uptake (cytoplasm) is performed for individual cells by the concomitant use of bright field illumination and dark field-UV fluorescent illumination. See, Hayashida *et al.*,  
30 *J. Biol. Chem.* 6:271(36):21985-21992 (1996).



The studies described in this example tested activity in BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

5

**Example 27: Stimulation of Endothelial Migration**

This example will be used to explore the possibility that the BMP polypeptide may stimulate lymphatic endothelial cell migration.

Endothelial cell migration assays are performed using a 48 well  
10 microchemotaxis chamber (Neuroprobe Inc., Cabin John, MD; Falk, W., et al., J. Immunological Methods 1980;33:239-247). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8  $\mu$ m (Nucleopore Corp. Cambridge, MA) are coated with 0.1% gelatin for at least 6 hours at room temperature and dried under sterile air. Test substances are diluted to appropriate concentrations in M199 supplemented with  
15 0.25% bovine serum albumin (BSA), and 25  $\mu$ l of the final dilution is placed in the lower chamber of the modified Boyden apparatus. Subconfluent, early passage (2-6) HUVEC or BMEC cultures are washed and trypsinized for the minimum time required to achieve cell detachment. After placing the filter between lower and upper chamber,  $2.5 \times 10^5$  cells suspended in 50  $\mu$ l M199 containing 1% FBS are seeded in  
20 the upper compartment. The apparatus is then incubated for 5 hours at 37°C in a humidified chamber with 5% CO<sub>2</sub> to allow cell migration. After the incubation period, the filter is removed and the upper side of the filter with the non-migrated cells is scraped with a rubber policeman. The filters are fixed with methanol and stained with a Giemsa solution (Diff-Quick, Baxter, McGraw Park, IL). Migration is  
25 quantified by counting cells of three random high-power fields (40x) in each well, and all groups are performed in quadruplicate.

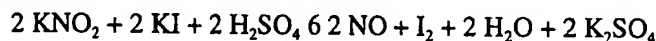
The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy),  
30 agonists, and/or antagonists of the BMP polypeptide.

**Example 28: Stimulation of Nitric Oxide Production by Endothelial Cells**

Nitric oxide released by the vascular endothelium is believed to be a mediator of vascular endothelium relaxation. Thus, BMP polypeptide activity can be assayed by determining nitric oxide production by endothelial cells in response to BMP polypeptide.

Nitric oxide is measured in 96-well plates of confluent microvascular endothelial cells after 24 hours starvation and a subsequent 4 hr exposure to various levels of a positive control and BMP polypeptide. Nitric oxide in the medium is determined by use of the Griess reagent to measure total nitrite after reduction of nitric oxide-derived nitrate by nitrate reductase. The effect of BMP polypeptide on nitric oxide release is examined on HUVEC.

Briefly, NO release from cultured HUVEC monolayer is measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments Inc.) (1049). Calibration of the NO elements is performed according to the following equation:



The standard calibration curve is obtained by adding graded concentrations of  $\text{KNO}_3$  (0, 5, 10, 25, 50, 100, 250, and 500 nmol/L) into the calibration solution containing KI and  $\text{H}_2\text{SO}_4$ . The specificity of the Iso-NO electrode to NO is previously determined by measurement of NO from authentic NO gas (1050). The culture medium is removed and HUVECs are washed twice with Dulbecco's phosphate buffered saline. The cells are then bathed in 5 ml of filtered Krebs-Henseleit solution in 6-well plates, and the cell plates are kept on a slide warmer (Lab Line Instruments Inc.) To maintain the temperature at 37°C. The NO sensor probe is inserted vertically into the wells, keeping the tip of the electrode 2 mm under the surface of the solution, before addition of the different conditions. S-nitroso acetyl penicillamin (SNAP) is used as a positive control. The amount of released NO is expressed as picomoles per  $1 \times 10^6$  endothelial cells. All values reported are means of four to six measurements in each group (number of cell culture wells). See, Leak *et al. Biochem. and Biophys. Res. Comm.* 217:96-105 (1995).

The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

5

**Example 29: Effect of the BMP Polypeptide on Cord Formation in Angiogenesis**

Another step in angiogenesis is cord formation, marked by differentiation of endothelial cells. This bioassay measures the ability of microvascular endothelial cells to form capillary-like structures (hollow structures) when cultured *in vitro*.

- 10 CADMEC (microvascular endothelial cells) are purchased from Cell Applications, Inc. as proliferating (passage 2) cells and are cultured in Cell Applications' CADMEC Growth Medium and used at passage 5. For the *in vitro* angiogenesis assay, the wells of a 48-well cell culture plate are coated with Cell Applications' Attachment Factor Medium (200 µl/well) for 30 min. at 37°C.
- 15 CADMEC are seeded onto the coated wells at 7,500 cells/well and cultured overnight in Growth Medium. The Growth Medium is then replaced with 300 µg Cell Applications' Chord Formation Medium containing control buffer or the BMP polypeptide (0.1 to 100 ng/ml) and the cells are cultured for an additional 48 hr. The numbers and lengths of the capillary-like chords are quantitated through use of the
- 20 Boeckeler VIA-170 video image analyzer. All assays are done in triplicate.

- Commercial (R&D) VEGF (50 ng/ml) is used as a positive control. b-estradiol (1 ng/ml) is used as a negative control. The appropriate buffer (without protein) is also utilized as a control. The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the
- 25 exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

**Example 30: Angiogenic Effect on Chick Chorioallantoic Membrane**

- Chick chorioallantoic membrane (CAM) is a well-established system to
- 30 examine angiogenesis. Blood vessel formation on CAM is easily visible and quantifiable. The ability of the BMP polypeptide to stimulate angiogenesis in CAM

can be examined. Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix*) are incubated at 37.8°C and 80% humidity. Differentiated CAM of 16-day-old chick and 13-day-old quail embryos is studied with the following methods. On Day 4 of development, a window is made into the egg  
5 shell of chick eggs. The embryos are checked for normal development and the eggs sealed with cellotape. They are further incubated until Day 13. Thermanox coverslips (Nunc, Naperville, IL) are cut into disks of about 5 mm in diameter. Sterile and salt-free growth factors are dissolved in distilled water and about 3.3 mg/5 ml are pipetted on the disks. After air-drying, the inverted disks are applied on  
10 CAM. After 3 days, the specimens are fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 M sodium cacodylate buffer. They are photographed with a stereo microscope [Wild M8] and embedded for semi- and ultrathin sectioning as described above. Controls are performed with carrier disks alone.

The studies described in this example tested activity in the BMP protein.  
15 However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

### **Example 31: Angiogenesis Assay Using a Matrigel Implant in Mouse**

20 *In vivo* angiogenesis assay of the BMP polypeptide measures the ability of an existing capillary network to form new vessels in an implanted capsule of murine extracellular matrix material (Matrigel). The protein is mixed with the liquid Matrigel at 4 degree C and the mixture is then injected subcutaneously in mice where it solidifies. After 7 days, the solid "plug" of Matrigel is removed and examined for the  
25 presence of new blood vessels. Matrigel is purchased from Becton Dickinson Labware/Collaborative Biomedical Products.

When thawed at 4 degree C the Matrigel material is a liquid. The Matrigel is mixed with the BMP polypeptide at 150 ng/ml at 4 degree C and drawn into cold 3 ml syringes. Female C57Bl/6 mice approximately 8 weeks old are injected with the  
30 mixture of Matrigel and experimental protein at 2 sites at the midventral aspect of the

abdomen (0.5 ml/site). After 7 days, the mice are sacrificed by cervical dislocation, the Matrigel plugs are removed and cleaned (i.e., all clinging membranes and fibrous tissue is removed). Replicate whole plugs are fixed in neutral buffered 10% formaldehyde, embedded in paraffin and used to produce sections for histological examination after staining with Masson's Trichrome. Cross sections from 3 different regions of each plug are processed. Selected sections are stained for the presence of vWF. The positive control for this assay is bovine basic FGF (150 ng/ml). Matrigel alone is used to determine basal levels of angiogenesis.

The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

#### **Example 32: Rescue of Ischemia in Rabbit Lower Limb Model**

To study the in vivo effects of the BMP polypeptide on ischemia, a rabbit hindlimb ischemia model is created by surgical removal of one femoral arteries as described previously (Takeshita, S. *et al.*, *Am J. Pathol* 147:1649-1660 (1995)). The excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels originating from the internal iliac artery (Takeshita, S. *et al.* *Am J. Pathol* 147:1649-1660 (1995)). An interval of 10 days is allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. At 10 day post-operatively (day 0), after performing a baseline angiogram, the internal iliac artery of the ischemic limb is transfected with 500 mg naked expression plasmid encoding the BMP polypeptide by arterial gene transfer technology using a hydrogel-coated balloon catheter as described (Riessen, R. *et al.* *Hum Gene Ther.* 4:749-758 (1993); Leclerc, G. *et al.* *J. Clin. Invest.* 90: 936-944 (1992)). When DNA encoding the BMP polypeptide is used in the treatment, a single bolus of 500 mg of the BMP protein or control is delivered into the internal iliac artery of the ischemic limb over a period of 1 min. through an infusion catheter. On day 30, various parameters are measured in these rabbits: (a) BP ratio - The blood

pressure ratio of systolic pressure of the ischemic limb to that of normal limb; (b) Blood Flow and Flow Reserve - Resting FL: the blood flow during undilated condition and Max FL: the blood flow during fully dilated condition (also an indirect measure of the blood vessel amount) and Flow Reserve is reflected by the ratio of max FL: resting FL; (c) Angiographic Score - This is measured by the angiogram of collateral vessels. A score is determined by the percentage of circles in an overlaying grid that with crossing opacified arteries divided by the total number in the rabbit thigh; (d) Capillary density - The number of collateral capillaries determined in light microscopic sections taken from hindlimbs.

10       The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

15    **Example 33: Effect of the BMP Protein on Vasodilation**

      Since dilation of vascular endothelium is important in reducing blood pressure, the ability of the BMP polypeptide to affect the blood pressure in spontaneously hypertensive rats (SHR) is examined. Increasing doses (0, 10, 30, 100, 300, and 900 mg/kg) of the BMP polypeptide is administered to 13-14 week old  
20   spontaneously hypertensive rats (SHR). Data are expressed as the mean +/- SEM. Statistical analysis are performed with a paired t-test and statistical significance is defined as  $p < 0.05$  vs. the response to buffer alone.

      The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the  
25   activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

**Example 34: Rat Ischemic Skin Flap Model**

      The evaluation parameters include skin blood flow, skin temperature, and  
30   factor VIII immunohistochemistry or endothelial alkaline phosphatase reaction. BMP

protein expression, during the skin ischemia, is studied using in situ hybridization.

The study in this model is divided into three parts as follows:

- a) Ischemic skin
- b) Ischemic skin wounds
- 5 c) Normal wounds

The experimental protocol includes:

- a) Raising a 3x4 cm, single pedicle full-thickness random skin flap (myocutaneous flap over the lower back of the animal).
- b) An excisional wounding (4-6 mm in diameter) in the ischemic skin (skin-flap).
- 10 c) Topical treatment with the BMP polypeptide of the excisional wounds (day 0, 1, 2, 3, 4 post-wounding) at the following various dosage ranges: 1mg to 100 mg.
- d) Harvesting the wound tissues at day 3, 5, 7, 10, 14 and 21 post-wounding for histological, immunohistochemical, and in situ studies.

The studies described in this example tested activity in the BMP protein.

- 15 However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

#### **Example 35: Peripheral Arterial Disease Model**

- 20 Angiogenic therapy using the BMP polypeptide is a novel therapeutic strategy to obtain restoration of blood flow around the ischemia in case of peripheral arterial diseases. The experimental protocol includes:

- a) One side of the femoral artery is ligated to create ischemic muscle of the hindlimb, the other side of hindlimb serves as a control.
- 25 b) BMP protein, in a dosage range of 20 mg - 500 mg, is delivered intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-3 weeks.
- c) The ischemic muscle tissue is collected after ligation of the femoral artery at 1, 2, and 3 weeks for the analysis of the BMP protein expression and histology. Biopsy is also performed on the other side of normal muscle of the contralateral
- 30 hindlimb.

The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

5

**Example 36: Ischemic Myocardial Disease Model**

The BMP polypeptide is evaluated as a potent mitogen capable of stimulating the development of collateral vessels, and restructuring new vessels after coronary artery occlusion. Alteration of the BMP protein expression is investigated in situ. The experimental protocol includes:

10

a) The heart is exposed through a left-side thoracotomy in the rat.

Immediately, the left coronary artery is occluded with a thin suture (6-0) and the thorax is closed.

b) BMP protein, in a dosage range of 20 mg - 500 mg, is delivered

15 intravenously and/or intramuscularly 3 times (perhaps more) per week for 2-4 weeks.

c) Thirty days after the surgery, the heart is removed and cross-sectioned for morphometric and in situ analyzes.

The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

20

**Example 37: Rat Corneal Wound Healing Model**

This animal model shows the effect of the BMP polypeptide on neovascularization. The experimental protocol includes:

25

a) Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.

b) Inserting a spatula below the lip of the incision facing the outer corner of the eye.

30

c) Making a pocket (its base is 1-1.5 mm from the edge of the eye).



d) Positioning a pellet, containing 50ng- 5ug of the BMP polypeptide, within the pocket.

e) BMP polypeptide treatment can also be applied topically to the corneal wounds in a dosage range of 20mg - 500mg (daily treatment for five days).

5       The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

10    **Example 38: Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models**

***A. Diabetic db+/db+ Mouse Model.***

To demonstrate that the BMP polypeptide can accelerate the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness  
15    wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al.*, *J. Surg. Res.* 52:389 (1992); Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)).

20       The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/+m) littermates. Mutant diabetic (db+/db+) mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al.* *Proc. Natl. Acad. Sci. USA* 77:283-293 (1982)). Animals show polyphagia,  
25    polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al.*, *J. Immunol.* 120:1375 (1978); Debray-Sachs, M. *et al.*, *Clin. Exp. Immunol.* 51(1):1-7 (1983); Leiter *et al.*, *Am. J. of Pathol.* 114:46-55 (1985)).  
Peripheral neuropathy, myocardial complications, and microvascular lesions,  
30    basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al.*, *Exp. Neurol.* 83(2):221-232 (1984);

Robertson *et al.*, *Diabetes* 29(1):60-67 (1980); Giacomelli *et al.*, *Lab Invest.* 40(4):460-473 (1979); Coleman, D.L., *Diabetes* 31 (Suppl):1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al.*, *J. Immunol.* 120:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this model may be similar to the healing observed in human diabetes (Greenhalgh, *et al.*, *Am. J. of Pathol.* 136:1235-1246 (1990)).

Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and are 8 weeks old at the beginning of the study. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med.* 172:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and

vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

The BMP polypeptide is administered using at a range different doses of the  
5 BMP polypeptide, from 4mg to 500mg per wound per day for 8 days in vehicle.  
Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium  
pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for  
histology and immunohistochemistry. Tissue specimens are placed in 10% neutral  
10 buffered formalin in tissue cassettes between biopsy sponges for further  
processing. Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls)  
are evaluated:

1) Vehicle placebo control

2) The BMP polypeptide

15 Wound closure is analyzed by measuring the area in the vertical and  
horizontal axis and obtaining the total square area of the wound. Contraction is then  
estimated by establishing the differences between the initial wound area (day 0) and  
that of post treatment (day 8). The wound area on day 1 is 64mm<sup>2</sup>, the corresponding  
size of the dermal punch. Calculations are made using the following formula:

20 
$$[\text{Open area on day 8}] - [\text{Open area on day 1}] / [\text{Open area on day 1}]$$
Specimens  
are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned  
perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome.  
Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected  
wounds. Histologic examination of the wounds are used to assess whether the healing  
25 process and the morphologic appearance of the repaired skin is altered by treatment  
with BMP polypeptide. This assessment included verification of the presence of cell  
accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and  
epidermal maturity (Greenhalgh, D.G. *et al.*, *Am. J. Pathol.* 136:1235 (1990)). A  
calibrated lens micrometer is used by a blinded observer.

30 Tissue sections are also stained immunohistochemically with a polyclonal  
rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is

used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is  
5 demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the  
10 lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation. Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

#### ***B. Steroid Impaired Rat Model***

15 The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, S.M. Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahl, S.M.*et al.*, *J. Immunol.* 115: 476-481 (1975); Werb, Z. *et al.*, *J. Exp. Med.* 147:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting  
20 angiogenesis, decreasing vascular permeability ( Ebert, R.H., *et al.*, *An. Intern. Med.* 37:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991); Haynes, B.F. *et al.*, *J. Clin. Invest.* 61: 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M., "Glucocorticoids and wound  
25 healing", In: Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck, L.S. *et al.*, *Growth Factors.* 5: 295-304 (1991); Haynes, B.F., *et al.*, *J. Clin. Invest.* 61: 703-797 (1978); Wahl, S. M., "Glucocorticoids and wound healing", In:  
30 Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New

York, pp. 280-302 (1989); Pierce, G.F. *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 2229-2233 (1989)).

To demonstrate that the BMP polypeptide can accelerate the healing process, the effects of multiple topical applications of the BMP polypeptide on full thickness  
5 excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and are 9 weeks old at the beginning of the study. The healing response of rats is  
10 impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the  
15 Guidelines for the Care and Use of Laboratory Animals.

The wounding protocol is followed according to section A, above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with  
20 sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and  
25 gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if  
30 granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

The BMP polypeptide is administered using at a range different doses of the BMP polypeptide, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution. Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The  
5 wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Four groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) are evaluated:

- 10        1) Untreated group  
          2) Vehicle placebo control  
          3) The BMP polypeptide treated groups.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated  
15 by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm<sup>2</sup>, the corresponding size of the dermal punch. Calculations are made using the following formula:

$$[\text{Open area on day 8}] - [\text{Open area on day 1}] / [\text{Open area on day 1}]$$

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks  
20 are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin is improved by treatment with the BMP polypeptide. A calibrated lens micrometer is  
25 used by a blinded observer to determine the distance of the wound gap.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the  
30 activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

**Example 39: Lymphadema Animal Model**

The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of the BMP polypeptide in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks. Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue. Circumference and volumetric measurements are then made following injection of dye into paws.

Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

Using a microscope, muscles in back of the leg (near the semitendinosus and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then and ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while

leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous peak, which typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days are measured. The effect plasma proteins on lymphedema is determined and whether protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

**Circumference Measurements:** Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people then those 2 readings are averaged. Readings are taken from both control and edematous limbs.

**Volumetric Measurements:** On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

**Blood-plasma protein measurements:** Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and Ca<sup>2+</sup> comparison.

**Limb Weight Comparison:** After drawing blood, the animal is prepared for tissue collection. The limbs are amputated using a quillitine, then both experimental and control legs are cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint is disarticulated and the foot is weighed.

**Histological Preparations:** The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel,



dipped into cold methylbutane, placed into labeled sample bags at - 80EC until sectioning. Upon sectioning, the muscle is observed under fluorescent microscopy for lymphatics. The studies described in this example tested activity in the BMP protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

**Example 40: Suppression of TNF alpha-induced adhesion molecule expression by the BMP Polypeptide**

The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

Tumor necrosis factor alpha (TNF-a), a potent proinflammatory cytokine, is a stimulator of all three CAMs on endothelial cells and may be involved in a wide variety of inflammatory responses, often resulting in a pathological outcome.

The potential of BMP polypeptide to mediate a suppression of TNF-a induced CAM expression can be examined. A modified ELISA assay which uses ECs as a solid phase absorbent is employed to measure the amount of CAM expression on TNF-a treated ECs when co-stimulated with a member of the BMP family of proteins. To perform the experiment, human umbilical vein endothelial cell (HUVEC) cultures are obtained from pooled cord harvests and maintained in growth medium (EGM-2; Clonetics, San Diego, CA) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37 degree C humidified incubator containing 5% CO<sub>2</sub>.

HUVECs are seeded in 96-well plates at concentrations of  $1 \times 10^4$  cells/well in EGM medium at 37 degree C for 18-24 hrs or until confluent. The monolayers are subsequently washed 3 times with a serum-free solution of RPMI-1640 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and treated with a given  
5 cytokine and/or growth factor(s) for 24 h at 37 degree C. Following incubation, the cells are then evaluated for CAM expression.

Human Umbilical Vein Endothelial cells (HUVECs) are grown in a standard 96 well plate to confluence. Growth medium is removed from the cells and replaced with 90 ul of 199 Medium (10% FBS). Samples for testing and positive or negative  
10 controls are added to the plate in triplicate (in 10 ul volumes). Plates are incubated at 37 degree C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100  $\mu$ l of 0.1% paraformaldehyde-PBS(with  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ) is added to each well. Plates are held at 4°C for 30 mins. Fixative is then removed from the wells and wells are washed 1X  
15 with PBS(+Ca,Mg)+0.5% BSA and drained. Do not allow the wells to dry. Add 10  $\mu$ l of diluted primary antibody to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10  $\mu$ g/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed X3 with PBS(+Ca,Mg)+0.5%  
20 BSA.

Then add 20  $\mu$ l of diluted ExtrAvidin-Alkaline Phosphatase (1:5,000 dilution) to each well and incubated at 37°C for 30 min. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA. 1 tablet of p-Nitrophenol Phosphate pNPP is dissolved in 5 ml of glycine buffer (pH 10.4). 100  $\mu$ l of pNPP substrate in glycine buffer is added  
25 to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphatase in glycine buffer:  $1:5,000 (10^0) > 10^{-0.5} > 10^{-1} > 10^{-1.5}$ . 5  $\mu$ l of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100  $\mu$ l of pNNP reagent must then be added to each of the standard wells. The plate must be incubated at 37°C for 4h.  
30 A volume of 50  $\mu$ l of 3M NaOH is added to all wells. The results are quantified on a plate reader at 405 nm. The background subtraction option is used on blank wells

filled with glycine buffer only. The template is set up to indicate the concentration of AP-conjugate in each standard well [ 5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.


The studies described in this example tested activity in the BMP protein.

- 5 However, one skilled in the art could easily modify the exemplified studies to test the activity of polynucleotides encoding the BMP polypeptide (e.g., gene therapy), agonists, and/or antagonists of the BMP polypeptide.

Applicant's or agent's file reference number	PT012PCT	International application PCT	unassigned
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>3</u> , line <u>23</u>	
<b>B. IDENTIFICATION OF DEPOSIT</b> Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 10801 University Boulevard Manassas, Virginia 20110-2209 United States of America	
Date of deposit May 22, 1998	Accession Number 209889
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable)      This information is continued on an additional sheet <input type="checkbox"/>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<b>For receiving Office use only</b> <input checked="" type="checkbox"/> This sheet was received with the international application Authorized officer 	<b>For International Bureau use only</b> <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer

*What Is Claimed Is:*

1. An isolated nucleic acid molecule comprising a polynucleotide selected from the group consisting of:
  - (a) the polynucleotide shown as SEQ ID NO:X or the polynucleotide encoded by a cDNA included in ATCC Deposit No:Z;
  - (b) a polynucleotide encoding a biologically active polypeptide fragment of SEQ ID NO:Y or a biologically active polypeptide fragment encoded by the cDNA sequence included in ATCC Deposit No:Z;
  - (c) a polynucleotide encoding a polypeptide epitope of SEQ ID NO:Y or a polypeptide epitope encoded by the cDNA sequence included in ATCC Deposit No:Z;
  - (d) a polynucleotide capable of hybridizing under stringent conditions to any one of the polynucleotides specified in (a)-(c), wherein said polynucleotide does not hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence of only A residues or of only T residues.
2. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide comprises a nucleotide sequence encoding a soluble polypeptide.
3. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide comprises a nucleotide sequence encoding the sequence identified as SEQ ID NO:Y or the polypeptide encoded by the cDNA sequence included in ATCC Deposit No:Z.
4. The isolated nucleic acid molecule of claim 1, wherein the polynucleotide comprises the entire nucleotide sequence of SEQ ID NO:X or a cDNA included in ATCC Deposit No:Z..
5. The isolated nucleic acid molecule of claim 2, wherein the polynucleotide is DNA.

6. The isolated nucleic acid molecule of claim 3, wherein the polynucleotide is RNA.
7. A vector comprising the isolated nucleic acid molecule of claim 1.
8. A host cell comprising the vector of claim 7.
9. A recombinant host cell comprising the nucleic acid molecule of claim 1 operably limited to a heterologous regulating element which controls gene expression.
10. A method of producing a polypeptide comprising expressing the encoded polypeptide from the host cell of claim 9 and recovering said polypeptide.
11. An isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:
  - (a) the polypeptide shown as SEQ ID NO:Y or the polypeptide encoded by the cDNA;
  - (b) a polypeptide fragment of SEQ ID NO:Y or the polypeptide encoded by the cDNA;
  - (c) a polypeptide epitope of SEQ ID NO:Y or the polypeptide encoded by the cDNA; and
  - (d) a variant of SEQ ID NO:Y.
12. The isolated polypeptide of claim 11, comprising a polypeptide having SEQ ID NO:Y.
13. An isolated antibody that binds specifically to the isolated polypeptide of claim 11.

14. A recombinant host cell that expresses the isolated polypeptide of claim 11.
15. A method of making an isolated polypeptide comprising:
  - (a) culturing the recombinant host cell of claim 14 under conditions such that said polypeptide is expressed; and
  - (b) recovering said polypeptide.
16. The polypeptide produced by claim 15.
17. A method for preventing, treating, or ameliorating a medical condition, comprising administering to a mammalian subject a therapeutically effective amount of the polypeptide of claim 11 or the polynucleotide of claim 1.
18. A method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising:
  - (a) determining the presence or absence of a mutation in the polynucleotide of claim 1; and
  - (b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or absence of said mutation.
19. A method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising:
  - (a) determining the presence or amount of expression of the polypeptide of claim 11 in a biological sample; and
  - (b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or amount of expression of the polypeptide.
20. A method for identifying a binding partner to the polypeptide of claim 11 comprising:
  - (a) contacting the polypeptide of claim 11 with a binding partner; and

(b) determining whether the binding partner effects an activity of the polypeptide.



<110> Human Genome Sciences, Inc.

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<130> PT012.PCT

<140> Unassigned

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<150> 60/092,922

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Ile Ile Thr Val Ser Thr Asn Gly Ser Ile His Ser Pro Arg Phe Pro
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His Thr Tyr Pro Arg Asn Thr Val Leu Val Trp Arg Leu Val Ala Val
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Glu Glu Asn Val Trp Ile Gln Leu Thr Phe Asp Glu Arg Phe Gly Leu
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Gln His Glu Arg Ile Ile Thr Val Ser Thr Asn Gly Ser Ile His Ser		
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Pro Arg Phe Pro His Thr Tyr Pro Arg Asn Thr Val Leu Val Trp Arg		
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His Phe Leu Ala Arg Leu Ser Pro Gly		
290	295	

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/15783

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC(6) :C12P 21/00; C07H 21/04; C07K 14/46 US CL :435/69.1; 536/23.4; 530/350 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/69.1; 536/23.4; 530/350		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, MEDLINE search terms: BMP, TGF-beta		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database GenBank, US National Library of Medicine, (Bethesda, MD, USA), No. AA631149. 'Homo sapiens cDNA clone', 31 October 1997, Abstract.	1, 7, 8
X	US 5,723,318 A (YAMAGUCHI et al.) 03 March 1998, SEQ ID NO:34 (nucleotides 445-465), SEQ ID NO:35 (amino acids 117-123), claims 5-12, paragraph bridging columns 16-17.	1, 2, 5, 7-11, 14-16
A,P	WOZNEY et al. Novel regulators of bone formation: Molecular clones and activities. Science, 16 December 1988, Vol. 242, pages 1528-1534, see entire document.	1-12, 14-16

<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.	
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family
Date of the actual completion of the international search 22 OCTOBER 1999	Date of mailing of the international search report 17 NOV 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID S. ROMEO Telephone No. (703) 308-0196 JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/15783

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-12, 14-16

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING<sup>91</sup>

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 11-12 and 14-16, drawn to an isolated polynucleotide and the polypeptide encoded thereby.

Group II, claim(s) 13, drawn to an antibody.

Group III, claim(s) 17, to the extent that it is drawn to a method of administering a polypeptide.

Group IV, claim(s) 17, to the extent that it is drawn to a method of administering a polynucleotide.

Group V, claim(s) 18, drawn to a diagnostic method comprising detecting the presence of a mutation in a polynucleotide.

Group VI, claim(s) 19, drawn to a diagnostic method involving detecting the presence of a polypeptide.

Group VII, claim(s) 20, drawn to a method of detecting a compound that binds a polypeptide.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: In order for these inventions to have unity of invention it is necessary that the single inventive concept be a contribution over the prior art. However, GENBANK ACCESSION NO. AA631149 teaches an isolated polynucleotide capable of hybridizing to the polynucleotide as shown in SEQ ID NO:2, as recited in claim 1. Therefore, group I does not fulfill the requirements of unity of invention with respect to the claimed polynucleotides. Group I does share a special technical feature with any of the other groups because group I does not have special technical feature that is a contribution over the prior art. With regard to the application of PCT Rule 13, 37 CFR § 1.475 concerning unity of invention states: (d) If multiple products, processes of manufacture or uses are claimed, the first invention of the category first mentioned in the claims of the application and the first recited invention of each of the other categories related thereto will be considered as the main invention in the claims, see PCT Article 17(3)(a) and § 1.476(c). Pursuant to 37 CFR § 1.475(d), this authority considers that where multiple products and processes are claimed, the first recited product, method of making that product, and method of using that product, together with the first recited of each of the other inventions related thereto, shall constitute the main invention. Further, it considers that any subsequently recited products and/or methods constitute separate groups. Accordingly, groups II-VII constitute separate groups.